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The ecology and evolution of malaria:
Laboratory studies of *Plasmodium chabaudi* and its
rodent and insect hosts

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Abstract

In this thesis I investigated selective pressures that could shape the ecology and evolution of the malaria parasite and its mosquito host. I used the rodent malaria parasite *Plasmodium chabaudi*, laboratory mice and the lab adapted malaria vector *Anopheles stephensi*.

Key theory underpinning much of this work is the virulence trade-off hypothesis, where virulence (harm to host) is thought to be an unavoidable consequence of the parasites effort to optimise fitness. Implicit in this theory is that virulence is an intrinsic parasite trait, so that a virulent parasite would be relatively virulent in all host types. Using four genetically distinct *P. chabaudi* clones and four distinct host strains I found for the most part, this assumption to be true; the virulence phenotype of each of the four parasites was the same across the four host strains tested. I further used these genetically distinct *P. chabaudi* clones along with candidate malaria vaccine AMA-1 to study 1) vaccine efficacy against single and mixed clone infections and 2) the potential selective effects of vaccination. I found that vaccination reduced parasite density in a strain specific manner and that diversity at loci other than the target antigen influenced vaccine efficacy. In both naïve and vaccinated hosts, selection for increased virulence was observed in mixed genotype infections, but in no case did vaccination enhance this selective pressure. As *Plasmodium* evolution is intricately linked to vector population dynamics, I explored environmental conditions that may influence *Anopheles* fitness. Specifically, I investigated whether the amount of food *Anopheles* parents experienced influenced the success of their offspring. I found that the food level of both parents and offspring were important in *Anopheles* fitness. In particular, daughters from parents reared in low food conditions produced more eggs than daughters from parents reared in high food conditions.

In sum, my results indicate that the within-host environment of the malaria parasite can act as a selective agent. In addition, the environmental conditions experienced by *Anopheles* larvae could impact vector population dynamics. Altering the within-host environment of the parasite or the environment of its vector could dramatically alter the ecology and evolution of the malaria parasite.

1. General introduction

1.1. What is evolutionary biology?

Evolutionary biology is the study of organisms in an evolutionary framework. Central to evolutionary thinking is the concept of fitness. Fitness is an individual's representation in the gene pool, or its reproductive success. Each genotype in the population has its own relative fitness, which is determined by the success of others. Key questions in evolutionary ecology are therefore concerned with determining what strategies an organism employs to maximize its fitness.

This differs somewhat from the approach concerned with describing mechanisms behind phenomena. Ernst Mayr was one of the first to highlight the differences between these two approaches, noting that functional biologists were mainly concerned with asking 'how' types of questions, while evolutionary biologists were mainly concerned with asking 'why' types of questions (Mayr 1961). Using the phenomena of migration in birds, Pianka (1983) extends Mayr's example to explain that these two approaches differ by providing proximate and ultimate reasons behind events. For a physiologist he notes that migration may be due to decreasing day length (photoperiod) causing birds to take flight. In contrast, for the evolutionary biologist, the explanation for migration may be that the reduced winter mortality of migratory birds increased the number of their offspring compared to non-migratory birds. Over a long period of time, this results in the selection for migratory behaviour due to differential reproductive success. The physiologist's answer describes the mechanism, whereas the evolutionary ecologist describes a 'strategy'. These two approaches are not mutually exclusive and modern ecologists incorporate both mechanism and adaptation (e.g. Stearns 1999; Thierry 2005; Read & Clark 2006)

The application of this approach can traditionally be seen in fields including biodiversity and ecology, behaviour and social evolution, and life history evolution. These fields are largely concerned with understanding the forces that generate and maintain the high level of diversity we see in life. The field of biodiversity and ecology examines this variation from the molecular through to the ecosystem level. Behaviours including co-operation, conflict, commensalism and parasitism are the realm of behavioural evolutionary biologist. Life history evolution is concerned with the allocation of resources to growth, survival and reproduction.

More recently, this approach has been extended into the areas of health and disease with the emergence of the field of 'Darwinian medicine'. This approach uses the principles of evolution to explain the basis of disease (e.g. Williams & Nesse 1991; Stearns 1999; Stearns & Ebert 2001). Gaining an understanding of why parasites harm their hosts (virulence) has been greatly aided by this approach. Three broad frameworks for explaining virulence have been established; these being that virulence is coincidental, virulence is a parasite adaptation and lastly coevolutionary interactions determine virulence (Stearns 1999). Using infection with *Plasmodium* (the disease causing agent of malaria) as an example, the morbidity and mortality associated with a malaria infection is believed to arise as a correlated side effect of a fitness enhancing trait (Mackinnon & Read 2004b). For example, the anaemia associated with severe disease is a side effect of transmission enhancing asexual parasite multiplication. Ultimate explanations of virulence can differ from the proximate explanations of virulence. One such proximate explanation of virulence is that it is caused by rosetting; the process where infected red blood cells adhere to uninfected red blood cells, cause obstructions and tissue damage leading to severe disease (e.g. Rowe et al. 2002; Sherman et al. 2003; Kirchgatter & Del Portillo 2005).

Virulence is not the only malaria parasite trait or consequence of infection that evolutionary thinking has been applied to. It has been suggested that either adaptively or coincidentally, observed sex ratios, host topor and host anaemia could all be fitness enhancing traits (reviewed by Paul et al. 2003). For example, evidence suggests that varied sex allocation in malaria parasites is an adaptive trait to enhance transmission (e.g. Shutler & Read 1998; Paul et al. 2002a; Paul et al. 2002b; West et al. 2002; Reece et al. 2005). Specifically, the change in sex ratio from female bias to approximately 50:50 in immune environments, could increase the probability that male gametes encounter females (Reece & Read 2000; Paul et al. 2002a; West et al. 2002). Manipulation of mosquito behaviour could also enhance parasite fitness. Increasing vector probing, altering vector resources and increasing vector longevity, may all be fitness enhancing mechanisms that increase parasite fitness by ensuring increased transmission from one host to the next (Poulin 1994; Hurd et al. 1995; Koella et al. 1998; Koella 2005).

1.2. Predictions and the trade-off model

An advantage of using an evolutionary approach is the predictive powers that evolutionary theory can bestow. In the case of malaria, a theoretical basis has been used to predict selective pressures that may lead to the evolution of virulence, as well as factors maintaining virulence in a population. Central to this has been the use of the trade-off model where virulence is an unavoidable consequence of the parasite's effort to optimise its fitness. Parasites require extensive host exploitation to obtain transmission to the next host, but at the same time this exploitation increases the probability that the parasite will kill the host (Anderson & May 1982; Sasaki & Iwasa 1991; Antia et al. 1994; Ebert & Herre 1996; Read et al. 2002). By this logic, parasites that replicate too slowly will be cleared by the host before they reach maximal transmission (Antia et al. 1994), and parasites that replicate too quickly will not reach maximal transmission if they kill their hosts (Anderson & May 1982; Frank 1996). The result of this is the selection for parasites with intermediate levels of virulence, so that they are virulent enough to obtain transmission, but not so virulent that they kill their hosts before this occurs.

In infections consisting of more than one genetically distinct pathogen lineage, it has been suggested that increased virulence could evolve due to within-host competition (Levin & Pimentel 1981; Bremermann & Pickering 1983; Ewald 1983; Bonhoeffer & Nowak 1994; Nowak & May 1994; van Baalen & Sabelis 1995; Frank 1996; Mosquera & Adler 1998; Fox et al. 2004). In this situation prudent parasites suffer from great fitness losses in hosts simultaneously infected with faster growing (virulent parasite). This is because virulent parasites kill the hosts before prudent parasites have realised their fitness potential. Even though hosts death also reduces the fitness of virulent parasites, prudent parasites suffer disproportionately and are eliminated by natural selection. The fitness benefit of virulence is

enhanced transmission; by extracting more resources from the host (i.e. red blood cells), the parasite is able to make more transmissible forms. This process is commonly known as the tragedy of the commons (Hardin 1968). Alternatively, pathogens could compete in different ways, such that within-host competition does not result in increased virulence (Chao et al. 2000; Brown et al. 2002; Schjorring & Koella 2003; West & Buckling 2003).

1.3. Selection on malaria parasites

1.3.1. The *Plasmodium* genus

Four *Plasmodium* species infect humans: *P. falciparum*, *P. malaria*, *P. ovale*, and *P. vivax*. *P. falciparum* is generally considered the more severe human malaria, with the majority of malaria-associated mortality due to this species. Many more *Plasmodium* species infect reptiles, birds and other mammals. Experimentally, many of these have been used as models for human malaria, either in their natural host or laboratory adapted hosts. The major rodent malaria model systems are *P. berghei*, *P. chabaudi* and *P. yoelii* in laboratory mice.

1.3.2. Malaria intervention strategies

Malaria intervention strategies are usually designed to act against either the replicating stages or the transmission stages of the malaria parasite. Intervention strategies designed against the replicating asexual stage, which include most anti-malarial drugs and potential vaccines, are aimed at easing the suffering of an individual by reducing the parasite density of their infection. In contrast, transmission blocking intervention strategies, including vector control programs, some types of vaccines and bednets, aim to reduce the transmission of malaria without any direct benefit to an infected individual and thus are commonly called interventions for the common good (Kaslow 1997; Carter et al. 2000; Tsuboi et al. 2003).

From a biomedical perspective an ideal intervention strategy would be one that reduced the morbidity and mortality the host. However, from an evolutionary perspective an ideal strategy would reduce the transmission of the parasite to a level where it was unable to be maintained. In epidemiological terminology, the average number of new infections resulting from an established infection is its R_0 (reproductive number). For the successful eradication of any infection the R_0 must be reduced to below 1, so that no new infections are established from an existing one.

1.3.3. Potential selective effects of vaccination

Current efforts to develop a malaria vaccine are focused on either the pre-erythrocytic stage, the asexual blood stages (anti-replication) or the transmission stage of the malaria parasite (most recently reviewed in Targett 2005; Chiang et al. 2006; Hill 2006; Reed et al. 2006). Potential anti-replication vaccines include MSP-1 and AMA-1 antigens and are primarily aimed at reducing the parasite density within an individual. If this reduction is incomplete, hosts could still become infected by and transmit parasites. Theoretically, a side-effect of this type of scenario could include the evolution of increased virulence in the parasite population (Gandon et al. 2001). Conventional wisdom suggests that excessively virulent parasites kill their hosts and therefore themselves. Even in situations where mortality is low, it is the risk of mortality that is in trade-off against the benefits of virulence (taken to be parasite transmission success). When virulence is intrinsically linked with parasite density and transmission success, it is feasible to expect lesser virulent parasites to be removed from the parasite population more quickly than virulent types. Critically, this selection depends on the type of vaccine. Anti infection and transmission – blocking vaccines will not favour increased virulence, as the costs associated with increased virulence are maintained (Read et al. 2004). The combination of antigens from different stages of the malaria life cycle is one approach that has the potential to

limit selection for increased virulence. Including an asexual antigen in a vaccine targeting sporozoites, hepatocytes or transmission stages, a second line of defence would be provided and the spread of selected parasites could be reduced (Richie & Saul 2002).

Genetic diversity among and within the *Plasmodium* species has been a key concern in the development of an effective malaria vaccine. This concern stems from experimental evidence showing antibody specificity to candidate vaccine antigens in human serum, as well as the specificity of the immune response to candidate vaccine antigens in animal models (Crewther et al. 1996; Hodder et al. 2001; Mota et al. 2001; O'Donnell et al. 2001; Ekala et al. 2002; Healer et al. 2004; Polley et al. 2004; Cortes et al. 2005). In both single and mixed genotype malaria infections, the specificity of vaccine could alter the subset of genotypes circulating in a population. Considering mixed infections only, specificity could also alter the multiplicity of an infection, as well the competitive interactions between parasites.

Malaria infections often consist of more than one genotype, arising from the injection of a diverse inocula or from the injection of genotype before an existing one is cleared (Babiker et al. 1991; Babiker et al. 1999a; Paul et al. 1999; Smith et al. 1999; Tanner et al. 1999; Engelbrecht et al. 2000; Bruce & Day 2002; Henning et al. 2004). As the host constitutes an environment of limiting resources as well as an environment under immune attack, it also seems likely that these genotypes are in competition. Indirect evidence for this exists in *P. falciparum* infections (Daubersies et al. 1996; Mercereau-Puijalon 1996; Arnot 1998; Smith et al. 1999; Bruce et al. 2000), and direct experimental evidence of competitive interactions in the rodent malaria model *P. chabaudi* have been demonstrated across a range of host strains, parasite genotypes, multiplicity and parasite dose (Snounou et al. 1992; Taylor et al. 1997; de Roode et al. 2003; de Roode et al. 2004a; de Roode et al. 2004b; de Roode et al. 2005a; de

Roode et al. 2005b; Bell et al. 2006; Raberg et al. 2006). From the above experiments the general phenomenon has emerged that in *P. chabaudi*, virulent genotypes are competitively superior to less virulent genotypes in mixed infections (de Roode et al. 2005b; Bell et al. 2006). This is consistent with the assumptions of many models of the evolution of virulence (Levin & Pimentel 1981; Bremermann & Pickering 1983; Ewald 1983; Bonhoeffer & Nowak 1994; Nowak & May 1994; van Baalen & Sabelis 1995; Frank 1996; Mosquera & Adler 1998; Fox et al. 2004); so that all else being equal mixed infections create a selective environment favouring more virulent strains than favoured in single clone infections.

To date, the study of competition in the rodent model has been restricted to naïve hosts. However, many people in malaria endemic areas are semi-immune and central to all vaccination programmes is the induction of immunity. Theoretically, it seems feasible that vaccine induced immunity could lead to either an increase or decrease in the level of competition. In practice, this depends on the mechanisms mediating competition. For example, if competition is largely resource based and anti-replication vaccines reduce parasite densities to levels where resources are not limiting, the level of competition may be relaxed. If competition is immune mediated and the anti-replicating vaccine is largely genotype specific against the more competitive genotype, competition could be relaxed. However, if competition is mediated via a transcending immune response, vaccine induced non-specific immunity may increase the competitiveness of an infection, leading to the preferential removal of the less competitive clone (Raberg et al. 2006)

Evidence of selective effects was recently observed in the clinical trial of an asexual blood stage vaccine, combination B. In this trial selection for infections that differed from the vaccine type occurred (Genton et al. 2002). The combination B vaccine comprises of the

merozoite surface protein (MSP-1), the 3D7 allele of MSP-2 and the ring infected erythrocyte surface antigen (RESA). During the follow up period of the vaccine trial, the prevalence of the 3D7 allele but not the alternate FC27 allele was reduced. As the FC27 type allele is also considered to result in more virulent infections (Engelbrecht et al. 1995), it is actually unclear as to whether vaccine escape was due to genetic polymorphism (as is generally assumed) or whether intrinsic parasite virulence played a role. Either way, the outcome of the trial was the same and the concerns regarding the selective effects of vaccination are warranted.

1.4. Selection of *Anopheles* mosquitoes

There are approximately 3,500 species of mosquitoes grouped into 41 genera. Of these female anopheles mosquitoes (Diptera: Culicidae) are responsible for the transmission of all of the malarias¹. Between, 40-70 species are considered to transmit malaria, with *Anopheles gambiae* and *Anopheles funestus* transmitting the more severe species of malaria *Plasmodium falciparum*.

1.4.1. Mosquito intervention strategies

To date, many mosquito control strategies have involved targeting the larval and adult stages of the mosquito life cycle stages, with the common aim of decreasing the vector population density and disease transmission (Shiff 2002; Pates & Curtis 2005). In particular, the process of environmental management has the aim of reducing the number of breeding sites and the vector population by disrupting larval breeding sites through the use of oil spraying and water drainage (Phillips 2001; Bond et al. 2004; De Castro et al. 2004; Fillinger et al. 2004). Traditional control strategies involving the application of insecticides to the insides of dwellings specifically targets the resting adult vectors (McCarroll et al. 2000; Killeen et al.

¹ <http://www.cdc.gov/Malaria/biology/mosquito/>

2002; Brey 2003) and, advances in this area have lead to the proposed use fungal biopesticides rather than chemical insecticides (Scholte et al. 2003; Blanford et al. 2005; Kanzok & Jacobs-Lorena 2006).

1.4.2. Mosquito fitness

Consisting of four distinct stages, *Anopheles* mosquitoes develop from eggs, through aquatic larval and pupae stages and finally emerge as full size adults. During each of these four life cycle stages the overall fitness of the mosquito is determined. Key life history traits during these stages include emergence time, size, fecundity and survival (Clements 1992; Charlwood 2003; Depinay et al. 2004). For both sexes, a reduced egg-to-adult emergence time is beneficial as it reduces generation time. Males with a shorter aquatic stage may also be at a further advantage if they emerge before females who become refractory to further mating. In contrast, a longer development time may increase body size, which is sometimes thought to be beneficial in mating success. For females, a classic trade-off between short development time and large adult size occurs, due to the positive correlation between size and fecundity (Clements 1992; Clements 1999).

These fitness components are influenced by genetic background, environmental conditions and interactions between the two. Key environmental factors include temperature, moisture and nutrition competition (Lyimo et al. 1992; Suwanchaichinda & Paskewitz 1998; Tun-Lin et al. 2000). Another environmental factor which is increasingly recognised to have a profound impact on individual success is the environmental conditions experienced by parents (Kirkpatrick & Lande 1989; Bernardo 1996; Rossiter 1996; Mousseau & Fox 1998; Wolf et al. 1998). Such transgenerational effects are a common form of phenotypic plasticity where an individual's characteristics are influenced by the environment or condition of its parent. If

present, mosquito control methods resulting in environmental modification of larval habitat, as well as strategies involving the large scale rearing and release of genetically modified or sterile males may benefit from an increased understanding of how parent and offspring larval rearing conditions interact to alter mosquito ecology and evolution.

1.5. Experimental aims

In this thesis I use an evolutionary approach to explore factors shaping the ecology and evolution of *Plasmodium* and *Anopheles*, using the rodent malaria *P. chabaudi* and laboratory reared *A. stephensi*. Each chapter is been written as a stand alone paper, with its own introduction. However, a summary of the experimental aims are as follows:

In chapter 2, I explore the applicability of evolution of virulence trade-off theory to the rodent malaria model system *P. chabaudi*.

In chapters 3 and 4, I investigate the potential selective effects of an asexual malaria vaccine on *Plasmodium*. Specifically, I ask:

- a) How does parasite diversity and virulence influence vaccine efficacy?
- b) Is competition increased or alleviated in immune animals?

In chapter 5, I investigate the potential of transgenerational effects to influence *Anopheles* population dynamics. Specifically I ask,

Do the larval habitats of *Anopheles* parents influence the key life history traits of emergence time, survival, blood meal size and fecundity of their offspring?

2. Host-by-parasite interactions for virulence and resistance in a malaria model system.

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2.1. Abstract

A rich body of theory on the evolution of virulence (disease severity) attempts to predict the conditions that cause parasites to harm their hosts, and a central assumption to many of these models is that virulence of a pathogen strain is stable across a range of host types. In contrast, a largely non-overlapping body of theory on coevolution assumes that the fitness effects of parasites on hosts is not stable across host genotype, but instead depends on host genotype by parasite genotype interactions. If such genetic interactions largely determine virulence, it becomes difficult to predict the strength and direction of selection on virulence. In this study I tested for host-by-parasite interactions in a medically relevant vertebrate disease model: the rodent malaria parasite *Plasmodium chabaudi* in laboratory mice. I found that parasite and particularly host main effects explained most of the variance in virulence (anaemia and weight loss), resistance (parasite burden) and transmission potential. Host-by-parasite interactions were of limited influence, but nevertheless had significant effects. This raises the possibility that host heterogeneity may affect the rate of any parasite response to selection on virulence. This study of rodent malaria is one of the first tests for host-by-parasite interactions in any vertebrate disease; host-by-parasite interactions typical of those assumed in coevolutionary models were present, but were by no means pervasive.

2.2. Introduction

Pathogen evolution can be a key obstacle in the development of effective disease control programmes. Drug resistance is the most obvious example (e.g. Nkengasong et al. 2004; Talisuna et al. 2004; e.g. Olliaro 2005; Tripathi et al. 2005), but the possibility that pathogen virulence (here defined as harm to the host following infection) could also evolve is beginning to attract the attention of evolutionary biologists (Ewald 1994; Gandon et al. 2001; Dieckmann et al. 2002). For the most part, virulence evolution has been studied using parasite-centred models, where the direction of selection acting on parasite-encoded virulence is modelled as an optimality problem (e.g. Bremermann & Pickering 1983; May & Anderson 1983; Frank 1996; Andre et al. 2003; Choo et al. 2003; Day & Proulx 2004). This has led to ideas of virulence management (Dieckmann et al. 2002) and specific predictions about, for instance, public health strategies which could prompt the evolution of benign parasites (Ewald 1994) or create the conditions which would favour more virulent pathogens (Gandon et al. 2001).

This parasite-centric approach to virulence evolution, which has attracted some controversy (e.g. Soubeyrand & Plotkin 2002; Ebert & Bull 2003), coexists uneasily alongside a largely independent body of work on host-parasite coevolution which emphasises that parasite and host genotypes together determine virulence (e.g. Ebert & Hamilton 1996; Woolhouse et al. 2002). Part of the unease is semantic: the term virulence is used in some of the coevolutionary literature (e.g. gene-for-gene and matching allele models) to mean the ability to infect a host, rather than as harm *per se*. However, even with these models and certainly for more general notions of on-going host-parasite arms races, the harm done to hosts following infection (the definition of virulence I use here) is a key source of selection. A more important difference between the two literatures is the nature of genetic control of disease severity. Coevolutionary arguments necessarily posit that harm is determined by interactions between parasite and host

genotypes, with particular strains being harmful in some host genotypes and benign on others. The specificity of these host-parasite interactions provides the genetic basis of alleged ongoing coevolutionary dynamics. The evolution of virulence literature ignores such genotype-by-genotype specificity and emphasises instead parasite encoded virulence.

These parasite-centric and coevolutionary views, which emphasize respectively, parasite main effects and host genotype by parasite genotype interactions, are not necessarily mutually exclusive. Optimality models typically assume that a given pathogen strain has a virulence phenotype that is stable across a range of host genotypes (e.g. Andre et al. 2003; Choo et al. 2003; Day & Proulx 2004; Lambrechts et al. 2006a) (Figure 2.1a). Assuming that virulence and transmission are positively correlated, as is often the case (e.g. Lipsitch & Moxon 1997; Mackinnon & Read 1999a), the transmission success and virulence of each parasite genotype could still vary, depending on for example, host genetic background or immune status. However, the relative impact of the different pathogen strains remains constant across host types (Figure 2.1b). In contrast, a variety of non-additive (host) genotype x (parasite) genotype interactions is also possible. In some cases, some parasite strains may cause more virulence than others, with this effect more pronounced in certain host genotypes than others (Figure 2.1c). Host-by-parasite interactions in coevolutionary models are such that a pathogen strain that causes high virulence in a particular host genotype could actually be the more benign strain in another genotype, commonly known as crossing of reaction norms (Figure 2.1d)

If such genotype-by-genotype interactions are widespread, it becomes increasingly difficult to predict evolutionary responses to selection on pathogen-encoded virulence determinants. If virulence in nature is largely a consequence of the genotype-by-genotype specificity of host-parasite interactions, it is difficult to imagine that parasite-centred optimality models of

pathogen virulence could provide much insight into evolutionary trajectories. Precisely analogous arguments can be made for resistance (host ability to control parasites). If genetic variation for resistance is mainly due to a main effect of host, then resistance evolution can be modelled as a simple host-centred optimality problem. If control of parasites is predominately a consequence of host-by-parasite interactions as in Figure 2.1d, it can not.

There has been some work on specificity in host-pathogen interactions for plant and invertebrate hosts (e.g. Carius et al. 2001; Ferrari & Godfray 2003), but almost none on medically relevant diseases in vertebrates; most studies have concentrated on either host variation (e.g. Abel & Dessein 1997; Amarante & Oliveira-Sequeira 2002; Mackinnon et al. 2002; de Roode et al. 2004b), or pathogen variation (Appleford & Smith 1997; Macedo & Pena 1998; Mackinnon et al. 2005), but not both. Yet, in both clinical and model malaria, it has long been recognised that both host factors (e.g. Stevenson et al. 1982; Burt 1999; Troye-Blomberg 2002) and parasite factors (e.g. Ariei et al. 2001; Read & Taylor 2001; Mackinnon & Read 2004b; Read et al. 2004; Kirchgatter & Del Portillo 2005) can affect disease outcome. Indeed, studies on human malaria have given us some of the best examples of host factors associated with resistance/susceptibility, including sickle cell anaemia (e.g. Williams et al. 2005) and particular MHC alleles (e.g. Segal & Hill 2003). Likewise, parasite-encoded phenotypes such as cytoadherence (Sherman et al. 2003) and rosetting (e.g. Rowe et al. 2002) are recognised to contribute to malaria severity. What is fundamentally lacking is any real understanding of the interactions between these host and parasite effects.

To address this, I performed a fully cross-factored experiment, using four parasite genotypes of *Plasmodium chabaudi* and four inbred mouse strains as hosts. I measured the virulence (severity) of the subsequent infections as well as within-host parasite abundance, which is a standard measure of host resistance (Fortin et al. 2001). So far as I am aware, this is one of the few times host-by-parasite interactions have been tested for in a medically relevant vertebrate system (see also Mackinnon et al. 2002). These results showed a combination of all possible scenarios (Figure 2.1a-d), depending on the measure of virulence or parasite success.

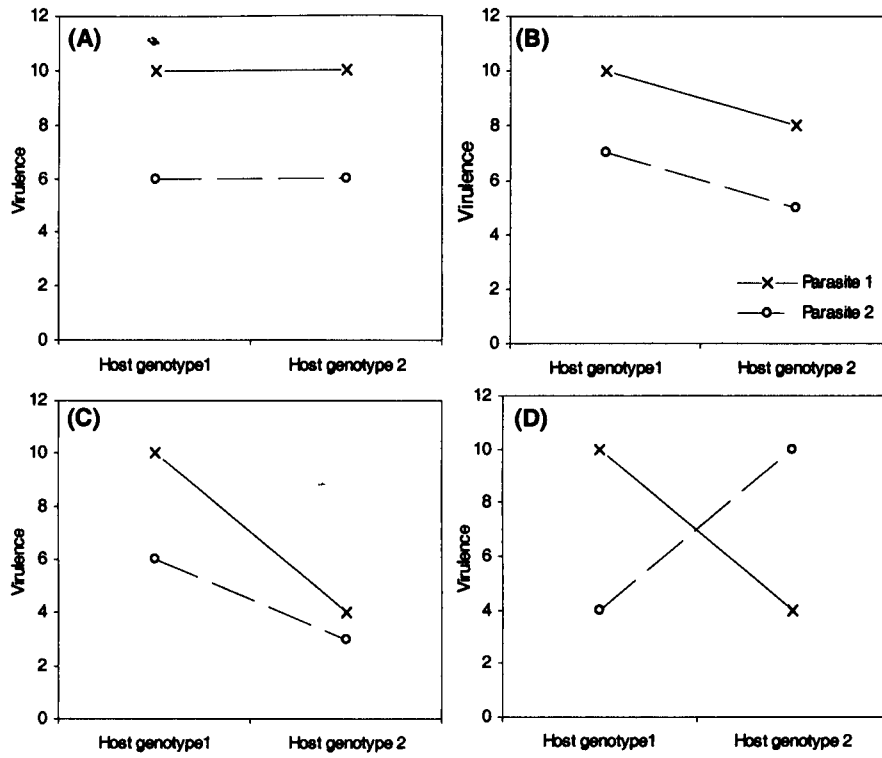


Figure 2.1

A schematic representation of host, parasite and host-by-parasite interaction effects, showing (a) a parasite main effect only, (b) additive parasite and host main effects, (c) non-additive host and parasite interactions without crossing reaction norms where pathogen differences are more apparent in one of the host genotypes and (d) host-by-parasite models.

2.3. Materials and methods

2.3.1. Parasites and Hosts

Plasmodium chabaudi isolates were collected from *Thamnomys rutilans* in the Central African Republic in 1969 and 1970. Genetically distinct parasite clones (Carter 1978), representing a range of virulence levels were then obtained from the isolates as described in Mackinnon and Read (1999). These clones are maintained as frozen stabulates, with the precise point in the clonal histories from which they come denoted with a subscript code. Below, I refer to them only using their letter codes for simplicity, but the clones were as follows: AJ₄₆₀₇, AS₁₁₉₁₈, CW₅₁₂, and ER₅₇₇.

Hosts were female mice, of inbred strains, CBA/CaOlaHsd, DBA/2OlaHsd, C57BL/6JolaHsd and NIH (Harlan England) aged 6-8 weeks. These hosts were chosen as they differ at the MHC (Lyon & Searle 1989) and control densities of *P. chabaudi* (clone AS) to varying degrees and are therefore known as more or less 'resistant' strains (Stevenson et al. 1982; Stevenson & Skamene 1986). Mice were fed on 41B maintenance diet (Harlan England) and drinking water was supplemented with 0.05% para-amino benzoic acid to aid parasite growth. Artificial light was provided from 05:30 to 17:30hrs. From hereon, hosts will be referred to as C57, CBA, DBA and NIH, with the term 'strain' used to denote mouse genotype and 'clone' to refer to parasite genotype.

2.3.2. Experimental design and inoculation of mice with parasites

The experiment was conducted in two replicate blocks four weeks apart. Both blocks consisted of 16 infected treatment groups (four clones x four strains), each with three replicate mice. Infections were initiated with an intra-peritoneal injection of 1×10^6 parasitised red blood cells. Inoculations were prepared by diluting infected blood from donor mice in a calf-serum

solution (50% heat inactivated Calf Serum; 50% Ringer solution [27mM KCL, 27mM CaCl_2 and 0.15 M NaCl], with 20 units of heparin /ml). Control mice received the same volume of uninfected red blood cells in calf-serum solution. Mice were sampled daily between days 3 and 21 post injection. Sampling involved determining mouse weight to an accuracy of 0.1g and red blood cell density using flow cytometry (Beckman Coulter). Thin smears from tail blood were fixed in methanol and stained in Giemsa to determine levels of asexual parasitaemia and gametocytaemia using microscopy.

2.3.3. Trait definition and statistical analysis

To determine the total number of asexual parasites and gametocytes present during the infection, I calculated the area under the curves for parasite and gametocyte density through time. Parasite densities and gametocyte densities are the products of the parasitaemia or gametocytaemia multiplied by red blood cell density. All density data were transformed using $[\log_{10} (\text{density} + 10)]$ to normalise the residuals. Only mice surviving until the end of the experiment were used in analysis of these data. I also determined the maximum parasite density as well as the number of days taken to reach the maximum. As all mice that died had declining parasite densities when they died, they were included in the maximum parasite density analysis. Three mice had standard asexual infection profiles, but produced no detectable gametocytes. As we had never previously observed infections with no gametocytes and as these three zero values generated enormous residuals the mice were removed from the gametocyte analysis so as to not violate statistical assumptions.

For measures of virulence I determined the ‘minimum weight’ and the ‘minimum red blood cell density’ reached during the experiment. Most models of virulence focus on the risk of death as a virulence measure, for which minimum weight and minimum red blood cell density

are correlates (Mackinnon & Read 2004b). It was assumed that all mice reach these minima prior to death. Therefore, even mice that died were included in the virulence analysis. Initial weight and initial red blood cell density were included as covariates where applicable and the density data was transformed using $[\log_{10}]$ to normalise the residuals.

I investigated the effect of host strain (four levels), parasite clone (four levels) and a host-by-parasite interaction on these variables using General Linear Models (Minitab 14, Minitab Inc., State College, PA, USA) or Proportional Hazards (JMP in 5.1, SAS Institute Inc., USA). For all our models I first fitted the maximal models including the main effects of host strain, parasite genotype and experimental block, a covariate (when relevant) and all two and three-way interactions. I then minimized the models by removing non-significant terms ($p > 0.05$), beginning with the highest-level interaction. As 'block' main effects are of little biological interest in their own right, I reported them only if they interacted significantly with the host-by-parasite interaction. I also investigated the relationships between asexual parasites, transmission stages and virulence, which are key relationships in evolutionary theory (Frank 1996), with regression analysis of the variables of 'total parasite density', 'total gametocyte density' and 'weight loss'.

2.4. Results

2.4.1. Parasite kinetics

The kinetics of parasite and gametocyte densities are illustrated in Figure 2.2. and virulence in Figure 2.3. Within all host strains, parasites increased in density followed by a dramatic reduction and then recrudescence (Figure 2.2.). Patterns of virulence were largely the inverse of the parasite densities, with an initial reduction in both weight and red blood cell density, followed by either a full or partial return to pre-infection levels (Figures 2.3). During the experiment, nine out of 96 mice died during the course of infection. Five of these hosts were of the NIH strain, with 3 infected with the AJ parasite clone and two with ER clone. Three further deaths occurred in CBA hosts with two of these hosts infected with AS and the other with the AJ. One further death occurred in host strain DBA with an AJ clone infection.

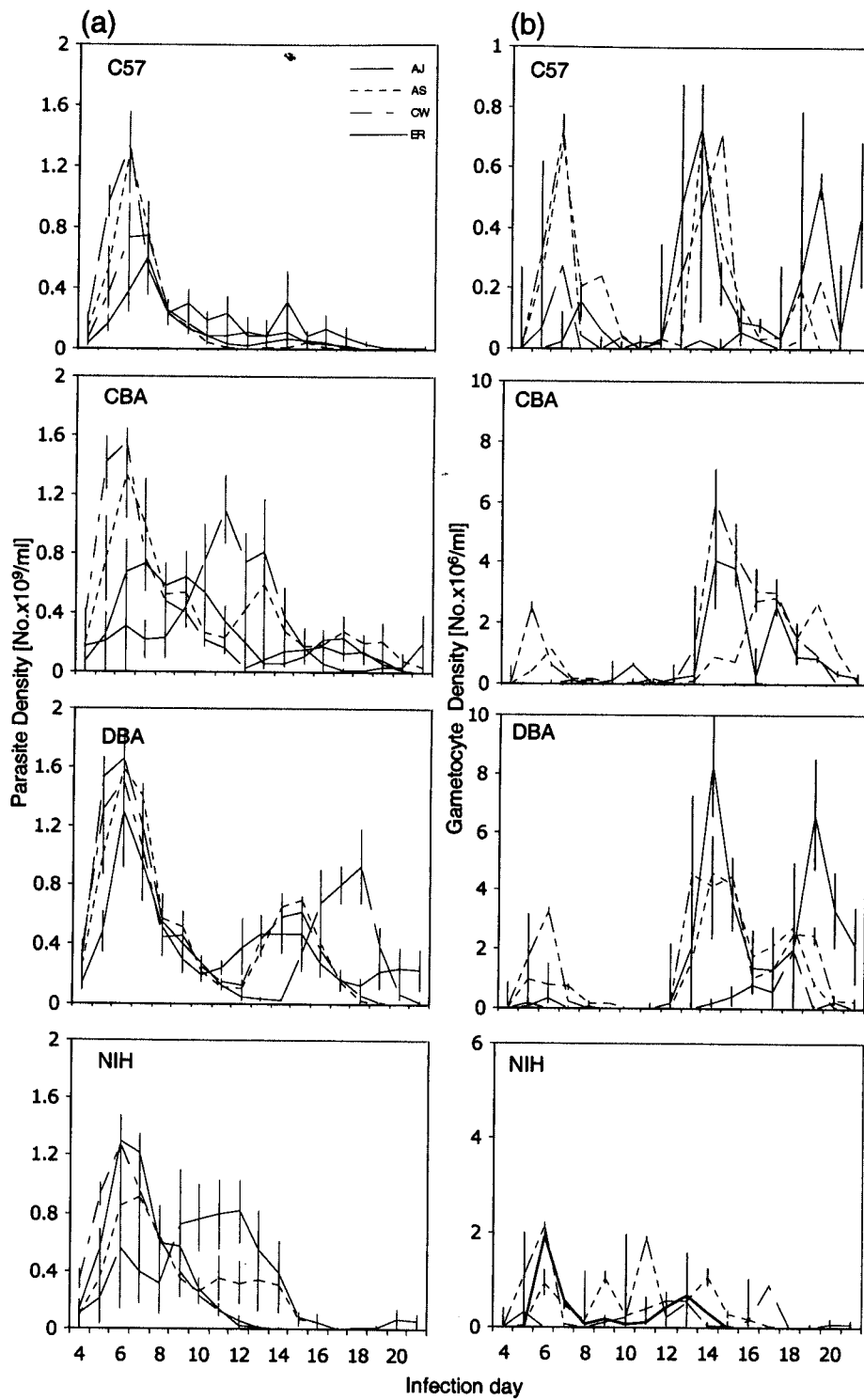


Figure 2.2. Legend on next page

Figure 2.2. Parasite (a) and gametocyte (b) densities through time, for each of the four *Plasmodium chabaudi* clones in each of the four host strains tested. Each line represents the mean parasite density for each parasite clone (AJ, AS, CW or ER) in each host strain (C57, CBA, DBA and NIH) with the associated standard error, averaged across two experiments. Each data point is an average up to six mice. Note different y-axis for gametocyte densities, which usually constitute <1% of all parasite.

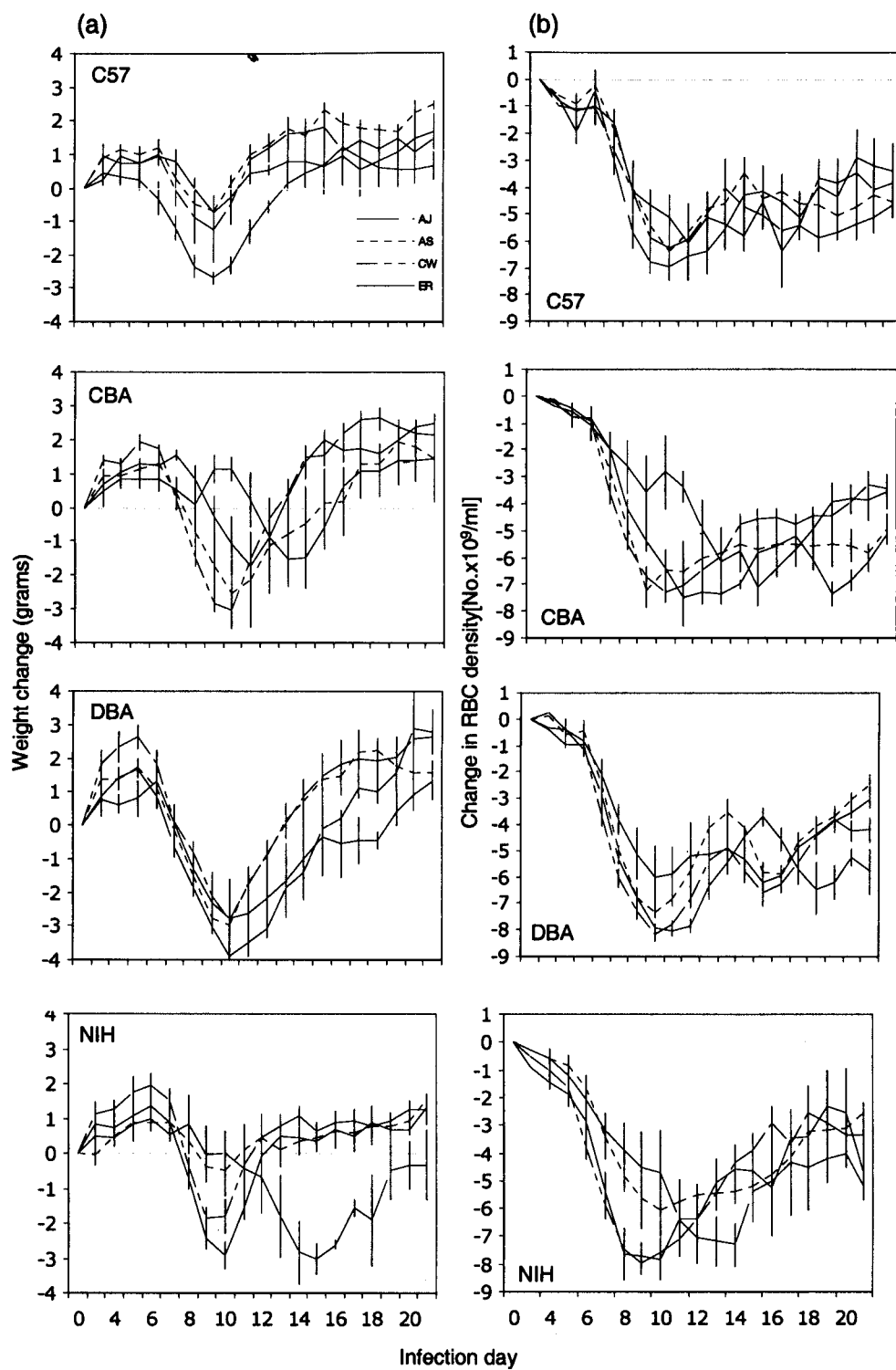


Figure 2.3. Legend on next page

Figure 2.3. Daily weight change (a) and red blood cell density change (b), from pre-inoculation values (horizontal lines). Each line represents the mean for each parasite clone in each host strain. Each data point is an average of up to six mice.

2.4.2 Parasite dynamics

Total parasite density in an infection was significantly affected by host and parasite main effects, as well as by a host-by-parasite interaction (Figure 2.4; Table 2.1a; $p=0.029$). However, across both blocks, clone AJ in host strain CBA displayed unusual dynamics by reaching its peak parasite density late in the infection (Figure 2.2). On its removal, the interaction term was no longer significant (Table 2.1b; $p=0.37$). In this and all further analyses of parasite dynamics, if any other single group was removed, the host-by-parasite interaction remained significant or marginally significant. I therefore report all results with and without the AJ-CBA treatment group (Table 2.1).

Within each host strain, a clone effect on parasite density could be detected only in strains CBA and DBA ($F_{3, 16}=11.6$, $p<0.01$; $F_{3, 16}=3.5$, $p=0.037$ respectively; C57: $F_{3, 16}=1.9$, $p=0.16$; NIH: $F_{3, 16}=0.97$, $p=0.43$). In host strain CBA, total parasite densities of parasite clones AJ and ER were lower than densities of AS and CW, while in host strain DBA, the total parasite density of clone AJ was greater than the others.

The average time taken to reach the maximum parasite density ranged between 5 – 11 days. This timing was influenced by main effects and a host-by-parasite interaction (Figure 2.4b, $p=0.024$). On the removal of the AJ-CBA treatment group, the timing of the peak parasite density was not affected by host or parasite main effects or an interaction (host: $p=0.47$; parasite: $p=0.08$; interaction: $p=0.09$). Total gametocyte density was also determined by a host-by-parasite interaction (Figure 2.4c; Table 2.1a; $p=0.04$). Removing the AJ-CBA treatment group the interaction was no longer significant (Table 2.1b; $p=0.29$). An effect of parasite clone on gametocyte density was detected within all host strains (C57: $F_{3, 17}=7.8$, $p<0.01$; CBA: $F_{3, 16}=72.8$, $p<0.001$; DBA: $F_{3, 15}=6.7$, $p<0.001$; NIH: $F_{3, 14}=0.1.9$, $p=0.025$).

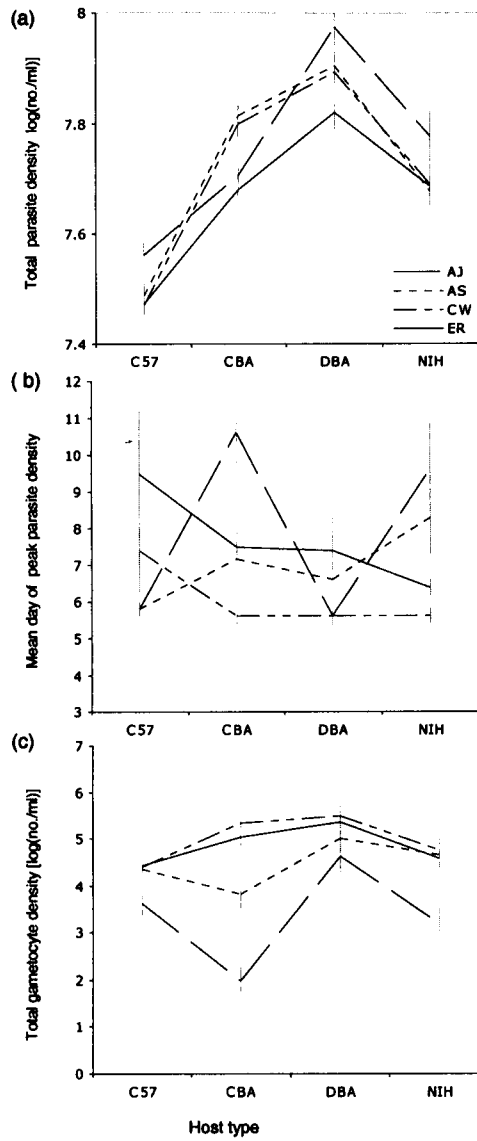


Figure 2.4. Host-by-parasite interaction plots in resistance and transmission potential, demonstrated by the (a) least square means of the total parasite density (b) mean day of peak parasite density and (c) least square means of the total gametocyte density, for each parasite clone (AJ, AS, CW or ER) in each host strain (C57, CBA, DBA and NIH) with the associated standard error.

Table 2.1 (a) The proportion of variance explained in resistance, transmission potential and virulence, by the main factors of parasite clone and host strain, as well as the host-by-parasite interaction in a *Plasmodium chabaudi* infection (b) The proportion of variance explained in resistance, transmission potential and virulence as above, excluding the parasite clone AJ in host strain CBA, which displayed unusual parasite kinetics. Note that the percentage explained by each factor does not always add to 100% as some of the variance is explained by other terms in the model. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0001$; ns=nonsignificant.

| | Parasite | | | Host | | | Host*Parasite | | |
|--------------------------|---------------------|-------|-----|----------------------|-------|-----|---------------------|-------|----|
| (a) | | | | | | | | | |
| Total Parasite Density | $F_{3,59} = 5.4$; | 3.4% | ** | $F_{3,59} = 135.1$; | 84.1% | *** | $F_{9,59} = 2.3$; | 4.2% | * |
| Total Gametocyte Density | $F_{3,56} = 36.4$; | 36.4% | *** | $F_{3,56} = 29.9$; | 30.2% | *** | $F_{9,56} = 2.1$; | 6.0% | * |
| Weight | $F_{3,71} = 4.8$; | 3.9% | ** | $F_{3,71} = 96.3$; | 78.4% | *** | $F_{9,71} = 3.7$; | 8.9% | ** |
| Red Blood Cell Density | $F_{3,72} = 3.9$; | 22.3% | * | $F_{3,72} = 7.0$; | 39.7% | ** | $F_{9,72} = 2.0$; | 34.2% | * |
| | | | | | | | | | |
| (b) | | | | | | | | | |
| Total Parasite Density | $F_{3,64} = 6.6$; | 4.4% | ** | $F_{3,64} = 130.7$; | 87.5% | *** | $F_{9,56} = 1.1$; | 1.8% | ns |
| Total Gametocyte Density | $F_{3,66} = 31.2$; | 47.4% | *** | $F_{3,66} = 32.3$; | 49.4% | *** | $F_{9,56} = 0.49$; | 2.1% | ns |
| Weight | $F_{3,67} = 2.5$; | 5.0% | * | $F_{3,67} = 99.2$; | 80.4% | *** | $F_{9,67} = 3.4$; | 7.4% | ** |
| Red Blood Cell Density | $F_{3,72} = 3.9$; | 17.2% | ** | $F_{3,72} = 11.6$; | 22.0% | *** | $F_{9,68} = 0.5$; | 7.8% | ns |

2.4.3 Virulence

Some of the virulence variation was due to host-by-parasite interactions (Table 1a, Figure 2.5; minimum weight; $p<0.01$, minimum red blood cell density; $p=0.048$). As with parasite densities, exclusion of the AJ-CBA treatment group resulted in a non-significant host-by-parasite interaction term for minimum red blood cell density (Table 1b; $p=0.83$), and with the removal of any other group the interaction still remained significant or marginally significant. In contrast, no single group accounted for the host-by-parasite interaction in minimum weight, which remained significant despite the removal of clone the AJ-CBA treatment group (Table 1b; $p<0.01$). Within each host strain, the effect of parasite clone on the minimum weight reached during the infection could only be detected in strains C57 and NIH ($F_{3, 16}=4.9$, $p=0.012$; $F_{3, 16}=8.4$, $p=0.001$ respectively; CBA: $F_{3, 18}=2.0$, $p=0.14$; DBA: $F_{3, 18}=0.46$, $p=0.7$). In both of these host strains, the parasite clones ranked from least to most virulent were: AS, CW, ER and AJ. Parasite differences in the minimum red blood cell density could be detected in host strains C57 and DBA ($F_{3, 16}=4.4$, $p=0.019$; $F_{3, 17}=3.2$, $p=0.049$ respectively; CBA: $F_{3, 18}=1.7$, $p=0.19$; NIH: $F_{3, 17}=1.3$, $p=0.30$) with the same rank order.

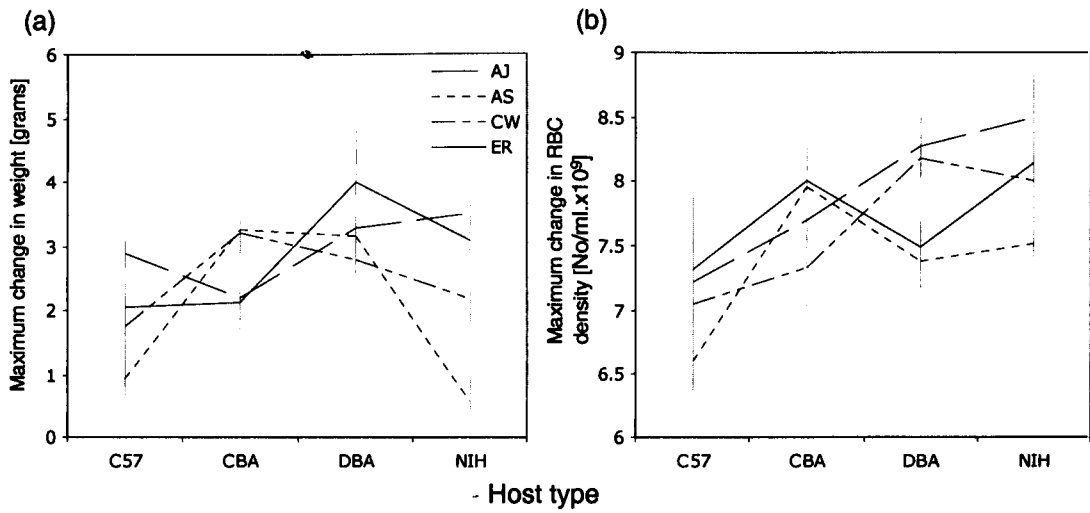


Figure 2.5 Host-by-parasite interactions in virulence, demonstrated by the (a) mean maximum change in (a) and the mean maximum change in red blood cell density (b) from pre-inoculation values, with the associated standard error, averaged across two experiments. Each data point is up to six mice for each parasite clone and host strain as above.

2.4.3 Relationships between traits

In parasite-centred models of virulence evolution, it is frequently assumed that parasite densities and/or transmission will be positively correlated with virulence (Frank 1996). I found that total gametocyte density was positively correlated with total parasite density (Figure 2.6a; $F_{1, 74}=24.4$, $p<0.001$), and was not influenced by a host-by-parasite interaction (host*parasite *total parasite density; $F_{9, 43}= 0.43$, $p=0.9$). Likewise, total parasite density was correlated with maximum weight loss in a linear positive manner (Figure 2.6b; $F_{1, 74}=32.8$, $p<0.001$), with no effect of an interaction (host*parasite*total parasite density; $F_{9, 43}= 0.88$, $p=0.5$). For both of these relationships, the 2-way interactions of host* total parasite density and parasite*total parasite density were non significant. There was no relationship between gametocyte density and weight loss (Figure 5c; $F_{1, 74}=1.0$, $p=0.31$) nor any host-by-parasite by weightloss interaction (host*parasite*weight loss; $F_{9, 43}= 1.0$, $p=0.39$). The 2-way interactions of host*weight loss and parasite*weight loss, were significant predictors of gametocyte density ($F_{9, 52}= 3.4$, $p=0.02$; $F_{9, 52}= 3.1$, $p=0.03$), so that relationships between virulence and transmission potential depend on both the host strain and the parasite clone, but not host-by-parasite interactions.

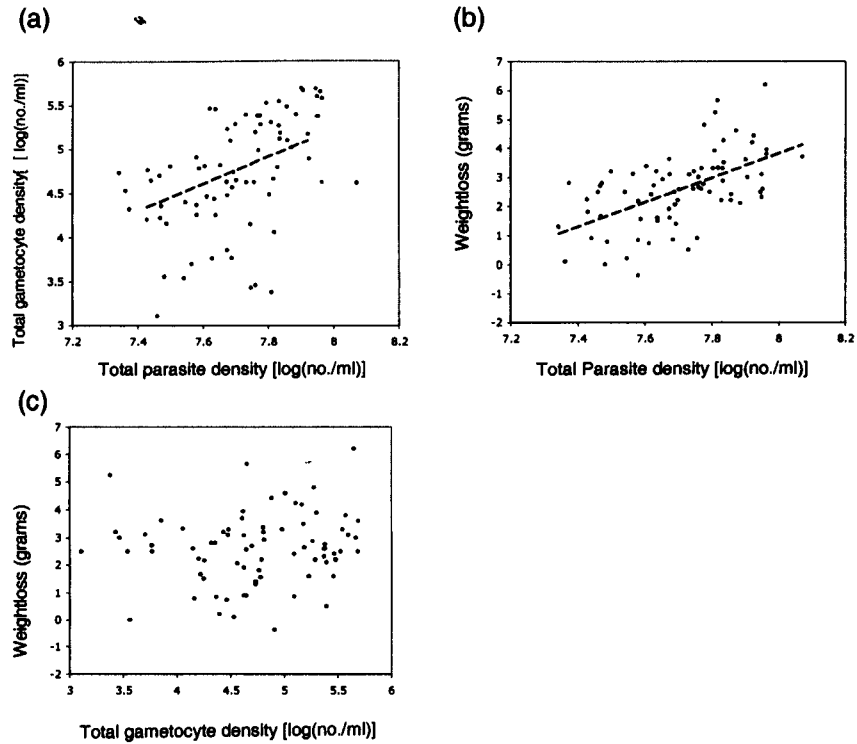


Figure 2.6 The relationships between (a) total parasite density and transmission potential (total gametocyte density) (b) virulence (weight loss) and total parasite density and (c) virulence and transmission potential (total gametocyte density), during *Plasmodium chabaudi* infection.

2.5 Discussion

In this study, I used the rodent malaria model system of *Plasmodium chabaudi* in laboratory mice to determine if host-by-parasite interactions were involved in determining the virulence, resistance and transmission potential of rodent malaria infections. The availability of a range *P. chabaudi* clones and a range of distinct hosts provided us with a unique opportunity to test biological assumptions implicit in both evolution of virulence and coevolutionary theoretical models in a medically relevant animal model.

I detected host genotype-by parasite genotype interactions, but found that they were generally of small effect size, primarily arising from the effects of one parasite clone in one particular host strain (Table 2.1a vs. 2.1b). Even with the inclusion of this treatment group, the interaction term explained only 4.2% and 6.0% of the variance in the total parasite density and gametocyte density respectively (Table 1a). For virulence, host-by-parasite interactions explained more of the variance: for minimum weight, the interaction explained 8.9% of the variance, while for red blood cell density, it explained 34.2% of the variance. Again though, much of this was due to one particular host-parasite combination (Table 2.1a vs. 2.1b). Overall, the interactions detected showed patterns akin to both types of interactions (Figures 2.1c and 2.1d). Parasite clone effects were at times more pronounced in certain host strains than in others, while crossing of reaction norms were also observed (Figures 2.4 and 2.5). The limited size of our interaction effects, however, clearly inhibits our ability to make precise comparisons to the simplified scenarios of Figure 2.1.

Experimental tests of virulence optimality theory and estimates of genotype-by-genotype interactions are very rare in vertebrate disease systems. In animal models of malaria, most work has been concentrated on different parasites in a single host genotype (Jarra & Brown

1989; Mackinnon & Read 1999a; Paul et al. 2004; de Roode et al. 2005b) , or a single parasite line in different host genotypes (e.g. Stevenson et al. 1982; Fortin et al. 2001; de Roode et al. 2004b). I am aware of only two other malaria studies that simultaneously examined both host and parasite genotypes; neither found host-parasite interaction and both found, as we did, that host genotype was a relatively more important determinant of both resistance and virulence than was parasite genotype. In the first study, three different mouse strains were infection with either a low virulence clone of *P. chabaudi* or a more virulent line derived from it by serial passage. For these two highly related lines, there was no evidence of host-parasite interactions for any of the variables examined (Mackinnon et al. 2002). The other study involved two unrelated clones in two strains of mice and again found no host-parasite interactions for virulence (de Roode et al. 2004b). The four treatment combinations in that experiment were also present in the experiments I report here and include the AJ-CBA combination we found to be a marked outlier responsible for most of the host-parasite interactions (Table 2.1, Figure 2.2). In that earlier study, the dynamics of clone AJ in CBA mice was much more in line with what we found for all of the host-parasite combinations. We have no explanation for the difference between the studies (I used the same laboratory, same mouse supplier, clonal lineages, diet, gender and approximate mouse weights), but we do note that the aberrant AJ-CBA dynamics we report occurred in both of our experimental blocks.

This experiment thus emphasises how the response to selection on parasite virulence could vary depending on host genotype: virulence variation was detectable in C57 mice but not, for example, in CBAs. As a consequence, it could be expected that the parasite response to selection on virulence would be more rapid in C57 mice than CBAs. Genetic and/or phenotypic host heterogeneity may therefore affect virulence evolution, as predicted by theory (e.g. Gandon & Michalakis 2000; Gandon et al. 2002; Ganusov et al. 2002). Selection

experiments involving serial passage of parasites have demonstrated that both naïve and semi-immune hosts can select for increased virulence, but passage through semi-immune hosts causes a more rapid virulence increase (Mackinnon & Read 2004a). These different rates of evolution could be explained if the expression of virulence differed between the naïve and semi-immune hosts, analogous to the differences I saw between host strains. Together these experiments highlight that predicting the effects of selection on parasites will require understanding of how host genotype and/or phenotype affect the expression of virulence.

Determining if semi-immunity affects the expression of virulence variation is not a relatively simple matter of repeating the experiments here using semi-immune animals. Immunity to malaria has a strain-specific component (e.g. Martinelli et al. 2005), so that choice of immunising strain becomes critical. Crucially, though, such experiments would allow us to determine if host immunity, including immunity generated by vaccination, will enhance the importance of parasite genotype relative to the effects of host genotype, as the determinant of virulence and resistance.

The experimental results I report here showed neither that highly specific host-by-parasite interactions dominated, nor that they were wholly absent. It may be that our choice of host strains or of parasite clones is unrepresentative of genetic diversity of *Plasmodium*-host interactions in the field. If a generality does exist, control strategies may be greatly aided by the knowledge that either parasite-centred models or coevolutionary models best capture malaria evolution. Of course, as with our experiments, the real world may involve aspects of both.

3. The impact of genetic diversity in *Plasmodium chabaudi* AMA-1 on vaccination with AMA-1 protein

3.1. Abstract

The potential for public health interventions to act as agents of selection is increasingly recognised. In particular, previous studies have shown that malaria anti-replication vaccines have the potential to select for both vaccine escape mutants and for increased virulence. In this study, I used a malaria model system of *Plasmodium chabaudi* in laboratory mice to determine the effect of parasite genetic diversity. I found that vaccination provided protection from virulence and that parasite density was reduced in a strain specific manner. Moreover, I found that diversity at loci other than the target antigen (which could include virulence determinants) influenced vaccine efficacy.

3.2. Introduction

Genetic diversity between and within *Plasmodium* species is a key issue for malaria control programs. Leading vaccine candidate antigens are frequently polymorphic in natural parasite populations, which may be a hindrance to vaccine efficacy if protection is conferred in a strain specific manner. One such leading blood stage antigen is the apical merozoite antigen (AMA-1) (Carvalho et al. 2002; Ballou et al. 2004; Greenwood et al. 2005). AMA-1 is thought to be involved in red blood cell invasion (Triglia et al. 2000; Mitchell et al. 2004; Healer et al. 2005) and is found on the rhoptry of the merozoite (Peterson et al. 1989). Across many *Plasmodium* species, AMA-1 has been found to lack the sequence repeats and size polymorphisms found in other blood stage candidate antigens (Marshall et al. 1989; Peterson et al. 1990; Waters et al. 1990; Kocken et al. 2000). What limited variation there is in AMA-1 is due to non randomly

dispersed point mutations (Peterson et al. 1989; Marshall et al. 1996). To date, the most divergent *P. falciparum* isolates have been found to differ at 37 nucleotides within the *ama-1* gene, resulting in 32 amino acid substitutions (Marshall et al. 1996).

In vitro studies using *P. falciparum* AMA-1 have indicated that these point mutations may be of immunological importance. In particular, merozoite invasion assays have shown that antibodies from rabbits immunised with recombinant *P. falciparum* AMA-1, as well as anti-AMA-1 antibodies from people in Papua New Guinea, inhibit parasite growth in a strain-specific manner (Hodder et al. 2001). Comparably, sera from animals immunised with the recombinant AMA-1 derived from *P. falciparum* laboratory clones FV0 and 3D7 showed greater inhibition of growth against themselves than against each other (Kennedy et al. 2002). Lastly, transgenic parasite lines that differ only in the hyper-variable region of domain 1 have also been shown to be inhibited in a specific manner, confirming relevance of polymorphism within the AMA-1 antigen (Healer et al. 2004).

The use of model systems is vital in the pre-clinical development of vaccines, and they have been used extensively to test delivery systems, adjuvants and immunisation protocols. However, the use of these models to test vaccine efficacy across a range of parasite genotypes has yet to be fully exploited. To date, rodent and non-human primate model systems have been used to determine the efficacy of the AMA-1 vaccine mainly against homologous whole parasite challenge (Deans et al. 1988; Crewther et al. 1996; Collins et al. 1997; Anders et al. 1998; Burns et al. 2003; Burns et al. 2004). In the limited number of studies using heterologous parasite challenge, mice immunised with recombinant AMA-1 derived from parasite line *P. chabaudi adami* [DS] were not protected against parasite line *P. chabaudi adami* [556KA] (Crewther et al., 1996; Anders et al., 1998). To our knowledge, no further

heterologous *in vivo* experiments have been reported in the testing of AMA-1 vaccine efficacy.

The consequence of a vaccine that imposes selection on the parasite population needs to be determined. The potential for such vaccine selection has already been demonstrated in the field trial of an asexual blood-stage vaccine (Combination B) (Genton et al. 2002). The combination B vaccine comprises of the merozoite surface protein MSP-1, the 3D7 allele of MSP-2 and the ring infected erythrocyte surface antigen (RESA). In this field trial, selection for infections that differed from the vaccine type occurred. During the follow up period it was shown that vaccination reduced the prevalence of the 3D7 allele of MSP-2, but not the alternate FC27 allele. Notably, previous studies suggest that this FC27 type allele results in more virulent infections (Engelbrecht et al. 1995). To date, the main focus of concern has centred on the problem of vaccine escape due to antigenic diversity (e.g. McLean 1995; Carvalho et al. 2002; Ballou et al. 2004; Greenwood 2005; Targett 2005). What is not clear from this field trial is whether selection on the parasite population was caused by vaccine escape due to immune specificity or whether the vaccine was less good at controlling more virulent infections. It is feasible that a combination of both processes will occur, and as we edge closer to a viable malaria vaccine it is becoming increasingly important to explore these possible outcomes.

In the experiments described here, I tested the effect of genetic diversity at the AMA-1 locus. To do this, I sequenced the *ama-1* gene of seven clones (termed AD, AJ, AQ, AS, AT, BC and CB) to determine how they differed from the vaccine derived clone DK. Based on both the sequence data and the virulence as described by Mackinnon & Read (1999a), I challenged vaccinated mice with a subset of heterologous clones

I found that vaccination provided general protection against weight loss and red blood cell loss. Vaccination also reduced parasite densities in a strain specific manner. Protection against the homologous challenge was significant but incomplete, with 50% of infections becoming patent. Among the heterologous parasites I found increased vaccine efficacy against parasites showing higher sequence similarity to the AMA-1 vaccine sequence and higher vaccine efficacy against the less virulent parasites.

3.3. Material and Methods

3.3.1. Parasites and Hosts

I studied eight genetically distinct *P. chabaudi* clones, denoted AD, AJ, AQ, AS, AT, BC, CB and DK (also known as 556KA). These isolates were originally collected from *Thamnomys rutilans* in the Central African Republic in 1969 and 1970, cloned as described by Mackinnon and Read (1999a) and stored in liquid nitrogen until use.

Hosts were inbred female C57BL/6JolaHsd mice aged 6-8 weeks (Harlan England). They were fed on 41B maintenance diet (Harlan UK) and their drinking water was supplemented with 0.05% para-amino benzoic acid to enhance parasite growth (Jacobs 1964) and kept in a 12L:12D cycle.

3.3.2. Sequencing of the AMA-1 alleles of *P. chabaudi*

Primers were designed to amplify the *ama-1* gene as two fragments. Primer sequences for fragment 1 were: Forward 5'CTTGGGTAATTGTTCCGA3' and reverse 5'GGTTTCCCAATCTTCACG3'. Primer sequences for fragment 2 were: Forward 5'GGGTCCAAGATATGTAG 3' and reverse 5' TGGTGTGTTGTGTGTGATGC3'. DNA was

amplified by PCR using Immolase™ DNA polymerase (Bioline). Sequencing reactions were performed using a Prism BigDye Terminator Cycle Sequence Kit Version 3.1 (ABI) and the University of Edinburgh SBS Sequencing Service ran the products of the reactions in-house on an ABI 3730 capillary sequencer. Sequences were analysed using DNASTar.

3.3.3. Experimental Design and Procedure

This experiment was performed as three experimental blocks (Table 3.1). In blocks 1 and 2, vaccinated and unvaccinated control mice were separated into 5 groups to be challenged with one of four heterologous strains AQ₁₉₆, AS₁₁₉₄₆, BC₂₀₈, and CB₁₁₀₆ or with the homologous strain DK₁₀₆. These five parasite clones were chosen out of the eight parasite clones sequenced due to their pre-determined virulence phenotype and by their sequence diversity. The parasite clones AQ, AS and BC (known to be of similar virulence) were shown to differ in their sequence similarity to the vaccine derived clone and were selected to test the role of genetic diversity on vaccine efficacy. Parasite clones AS and CB were known to differ in virulence but were shown have identical AMA-1 protein sequence and were therefore chosen to determine the role of virulence in vaccine efficacy. In experimental block 3, vaccinated and unvaccinated control mice were challenged with either AS or CB to clarify the role of virulence in vaccine efficacy.

Table 3. 1. The experimental design, showing the number of mice used in each block and for each parasite clone.

| | parasite DK | | parasite AS | | parasite CB | | parasite BC | | parasite AQ | |
|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|
| | control | vaccine | control | vaccine | control | vaccine | control | vaccine | control | vaccine |
| Block1 | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 3 | 3 |
| Block 2 | 8 | 5 | 8 | 5 | 8 | 5 | 8 | 5 | 8 | 5 |
| Block 3 | - | - | 5 | 5 | 6 | 6 | - | - | - | - |
| | 11 | 8 | 16 | 13 | 17 | 14 | 12 | 9 | 11 | 8 |

3.3.4. Vaccination

In all experimental blocks, recombinant AMA-1 derived from parasite clone DK was emulsified in Montanide ISA 720 (Seppic, Paris). Mice were injected intraperitoneally with 10 ug of protein in a 100ul emulsion. Boost immunisations were conducted 4 weeks after the primary immunisation. Control mice in experimental blocks 1 and 2, were injected with either 100ul of PBS, or 100ul of PBS emulsified in Montanide ISA 720. In experimental block 3, control mice were injected with 100ul PBS emulsified in Montanide ISA 720. The vaccination protocol was adapted from Crewther et al. (1996).

3.3.5. Parasite challenge and sampling

All infections were initiated with an intra-peritoneal injection of 1×10^5 parasitised red blood cells two weeks after the boost immunisation. Inoculations were prepared by diluting blood from donor mice in a medium of (50% heat inactivated Calf Serum; 50% Ringer solution [27mM KCL, 27mM CaCl₂ and 0.15 M NaCl]), 20 units of heparin /ml mouse blood).

Daily sampling involved weighing mice to an accuracy of 0.1g and estimating red blood cell density by flow cytometry (Beckman Coulter). In experimental blocks 1 and 2, thin tail blood smears were taken, fixed in methanol and stained in Giemsa to allow the counting of parasitaemia (proportion of red blood cells infected) using microscopy. When parasitaemia was high, I counted 500 red blood cells in at least four microscopic fields. With lower

parasitaemias I counted at least 20 microscopic fields (corresponding to at least 10 000 red blood cells), and calculated the average number of cells per field. Parasite density was calculated as the product of the parasitaemia and the red blood cell density. In experimental block 1, mice were sampled every day from day 3 to day 16 post infection (PI), while in experimental block 2 mice were sampled every day from day 3 to day 21 and then days 23 and 25 PI.

In experiment 3, the parasite density was determined by quantitative PCR (Cheesman et al. 2003; Bell et al. 2006). Five μ l samples of tail blood were collected into citrate saline every day from day 3 to day 21 and then days 23 and 25 PI. On the day of collection, samples were centrifuged at 13, 000 r.p.m, the supernatant removed and the pellet stored at -70°C for subsequent DNA extraction using Instagene Matric (BioRad.) Real time PCR cannot distinguish between asexual and gametocytes, however the relative density of gametocytes to asexual parasites is typically so low (<1%) that they do not affect the analysis or conclusions.

3.3.6. Trait definition and statistical analysis

Prior to analysis I defined and constructed the following summary traits to describe part of or all of the infection. For measures of virulence I determined the maximum red blood cell loss and maximum weight loss of the hosts from pre-inoculation levels (day -1). Maximum red blood cell loss data was transformed using \log_{10} (density) to meet normality and homogeneity of variance assumptions. Mice that died were included in the analysis of maximum weight loss and maximum red blood cell loss as it is assumed that they reached these maxima prior to death.

To calculate the numbers of parasites present during an infection, I calculated the area under the parasite density curve. All experimental blocks were used to determine total parasite densities during the acute phase of the infection, which occurs between days 3-16 post infection (PI). Mice that died during the experiment were included in acute phase analysis as all deaths occurred after the peak parasite density, and therefore after most of the acute phase parasites have been produced. In experimental blocks 2 and 3, parasite densities during the chronic phase (days 17- 25) were also calculated. Mice that died were not included in the chronic phase analysis. All density data was transformed using $[\log_{10}(\text{density} + 100)]$ to meet normality and homogeneity of variance assumptions.

I analysed all traits using General Linear Models (JMP in 5.1), with the explanatory variables being 'treatment', 'parasite clone' and where relevant 'experimental block'. Treatment had two levels (vaccinated and control); parasite clone had five levels in experimental blocks 1 and 2 (AQ, AS, CB, BC, DK) and two levels in block 3 (AS and CB) and experimental block had three levels (1, 2 and 3). For all the models I first fitted the maximal models and all two-way interactions where relevant. I then minimized the models by removing non-significant terms ($p > 0.05$), beginning with the highest-level interaction. As 'block' main effects are of little biological interest in their own right, I reported them only if they interacted significantly with the host-by-parasite interaction. Least square mean student t tests were used for pair-wise comparisons if the treatment*parasite clone interaction was significant

3.4. Results

3.4.1. Sequence diversity within the AMA-1 region of *P. chabaudi* clones

When comparing the nucleotide sequence of the heterologous parasite clones to the vaccine derived clone DK, I found AS (96.9%), BC (96.8%) and CB (96.9%) to be the most similar, with parasite clone AQ (93.9%) being the least. Among the heterologous clones, BC differed from AS and CB by one replacement mutation and AS and CB differed from each other by one silent mutation. The protein sequences of the parasites AS, BC and CB each differed from the protein sequence of DK at 18 residues, while AQ differed from DK at 30 (Figure 3.1.).

3.4.2. Mortality

During the experiment, 16 out of 119 (13.4%) mice died after the peak parasite density of the infection (Table 3.2.). Of these, 15 were infections with parasite clone CB, 10 of which were naïve hosts and 5 vaccinated. The other remaining death was in an unvaccinated host infected with parasite clone BC.

Table 3.2. The number of mice that died during the experiment, for each block and parasite clone. The last row gives the total number of mice that died per number infected.

| | parasite DK | | parasite AS | | parasite CB | | parasite BC | | parasite AQ | |
|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|
| | control | vaccine | control | vaccine | control | vaccine | control | vaccine | control | vaccine |
| Block1 | 0 | 0 | 0 | 0 | 3 | 1 | 0 | 0 | 0 | 0 |
| Block 2 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| Block 3 | - | - | 0 | 0 | 6 | 3 | - | - | - | - |
| TOTAL | 0/11 | 0/8 | 0/16 | 0/13 | 10/17 | 5/14 | 1/12 | 0/9 | 0/11 | 0/8 |

3.4.3. Virulence differences attributed to parasite clone, in naïve hosts only

In experimental blocks 1 and 2, control mice inoculated with PBS and control mice inoculated with PBS emulsified in montanide did not differ in red blood cell loss (treatment; $F_{1,45} = 1.2$, $p=0.27$) or weight loss (treatment; $F_{1,45} = 1.3$, $p=0.2$). These mice were therefore combined as a single control group.

Considering these control hosts only, differences in red blood cell loss, weight loss and host survival were influenced by the infecting parasite clone (Figure 3.2. solid lines only: red blood cell loss: $F_{4,59}=5.6$, $p<0.001$, weight loss: $F_{4,60}=9.5$, $p<0.001$, survival: $\chi^2=23.4$, $df=4$, $p<0.001$ respectively). Hosts infected with parasite clone DK had less red blood cell loss than the naïve hosts infected with the other parasite clones as on its removal from the analysis,

hosts infected with AS, BC, CB and AQ had similar levels of red blood cell loss ($F_{3, 49}=0.67$, $p=0.57$).

Parasite clones AS, BC and AQ did not differ in weight loss ($F_{2, 36}=0.9$, $p=0.38$). Grouping these three clones together and comparing them to parasite clone CB, I found CB infections to result in greater weight loss ($F_{1, 52}=7.8$, $p=0.007$). Parasite clone DK infections resulted in less weight loss than CB infections and the grouped AS, AQ, BC infections (DK vs. CB: $F_{1, 24}=26.9$, $p<0.0001$, DK vs. AS,AQ,BC: $F_{1, 46}=30.4$, $p<0.0001$). Parasite clones could be ranked from high to low virulence based on weight loss as: CB, (AS, AQ, BC), DK.

Specifically comparing parasite clones AS and CB, I found that unvaccinated hosts infected with parasite clone CB lost more weight and were more likely to die than unvaccinated hosts infected with AS ($F_{1, 26}=8.4$, $p=0.007$, $\chi^2=18.3$ df=1, $p<0.001$ respectively), although red blood cell loss was similar between these two groups ($F_{1, 26}=2.5$, $p=0.12$).

3.4.4. Virulence through time

The daily weight change and red blood cell dynamics are illustrated in Figure 3.2. In both vaccinated and control hosts, infections with parasite clone DK did not induce weight change, however weight loss was observed in infections with parasite clones AS, CB, BC and AQ, which was followed by a return to pre-infection levels. All mice apart from vaccinated hosts infected with parasite DK had an initial reduction in red blood cell density, followed by a full return to pre-infection levels.

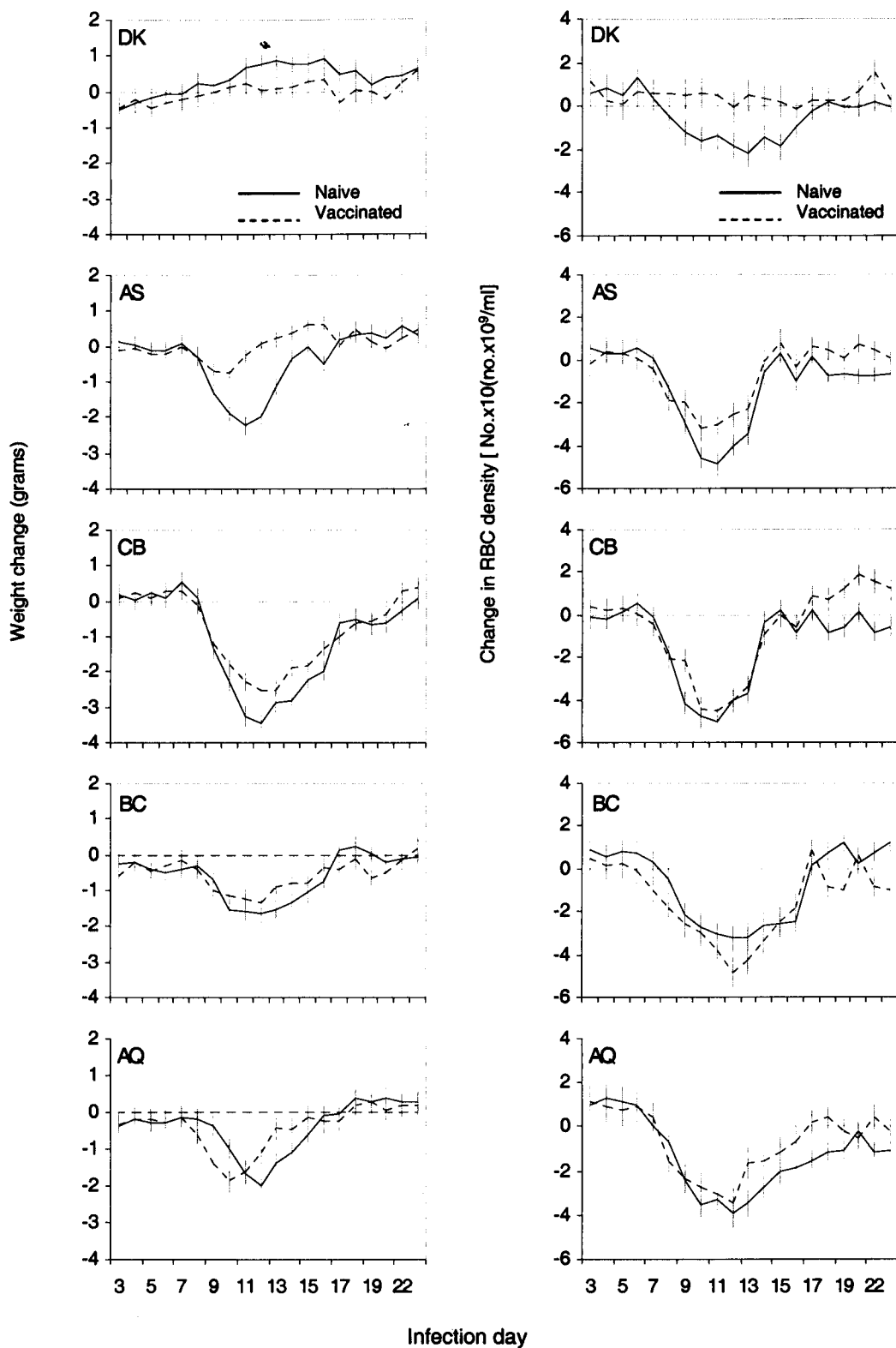


Figure 3.2. Legend on next page

Figure 3.2. Daily weight change and red blood cell density (RBC) from pre-infection levels. The solid line represents the unvaccinated hosts, while the dark dashed line represents the vaccinated. Each line represents the mean weight change or RBC change (average across experimental block \pm s.e.m.) of up to 17 mice. The grey dashed line represents the pre infection weight and red blood cell density.

3.4.5. Protective effect of vaccination on virulence

Vaccination provided protection against weight loss, with the level of protection the same across all parasite clones (Figure 3.3a: treatment: $F_{1, 111}=12.0$, $p<0.0001$, treatment*parasite: $F_{4, 107}=1.8$, $p=0.13$). Vaccination also provided protection from red blood cell loss, with the magnitude of this effect differing across parasite clones (Figure 3.3b: treatment*parasite: $F_{4, 106}=3.8$, $p=0.006$).

In contrast, no protective effect of vaccination from death was determined (treatment; $df = 1$ $\chi^2=1.98$ $p=0.16$, treatment*parasite; $df=4$ $\chi^2=0.74$ $p=0.94$). This may be due to the limited amount of death observed in this experiment. Across all blocks, eleven out of sixty seven (16.4%) unvaccinated hosts died compared to five out of forty two (11.9%) of vaccinated hosts.

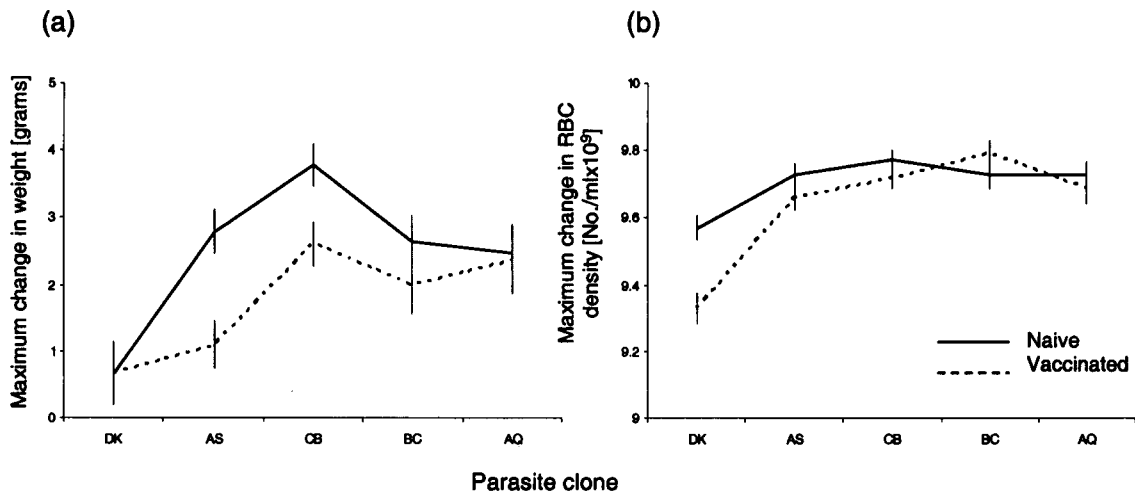


Figure 3.3. Maximum change in weight and (a) and red blood cell (RBC) densities (b) for the five parasite clones in naïve and vaccinated hosts. The solid line represents the naïve hosts, while the dashed line represents the vaccinated. Each line is the maximum weight change or RBC change (least-squares mean \pm SE from analysis) of up to 17 mice.

3.4.6. Parasite dynamics through time

The parasite kinetics are illustrated in Figures 3.4. All parasite infections followed similar dynamics, with parasite density increasing until days 7-9, followed by clearance. In parasite clones AS, CB and AQ, parasite density increases again in the chronic phase of the infection, a phenomenon known as recrudescence.

Control mice inoculated with PBS and control mice inoculated with PBS emulsified in montanide did not differ in acute phase density (treatment; $F_{1,44} = 1.3$, $p=0.26$) or chronic phase density (treatment; $F_{1,45} = 1.2$, $p=0.27$) and were therefore combined as a single control group.

During the acute phase, the anti-parasitic effect of vaccination differed among parasite clones (Figure 3.4. treatment*parasite; $F_{4,109}=25.3$, $p<0.001$). Vaccination conferred protection against hosts infected with the homologous parasite clone DK (DK vaccinated versus DK control; $t_{109}=11.6$, $p<0.001$) and the heterologous parasite clone AS (AS vaccinated versus AS control; $t_{109}=2.7$, $p=0.004$). Significant protection was not conferred against infections with parasite clones CB (CB vaccinated versus CB control; $t_{109}=0.89$, $p=0.18$), BC (BC vaccinated versus BC control; $t_{109}=0.6$, $p=0.25$) or AQ (AQ vaccinated versus AQ control; $t_{109}=0.38$, $p=0.35$). Note that least square mean student t tests were used for pair-wise comparisons. As these comparisons are based on the least square means determined from the full model, the degree's of freedom for these pair-wise tests equal the error degree's of freedom of the full model.

Removing the homologous parasite clone DK from the analysis, the effect of vaccination varied among the heterologous parasite clones (treatment*parasite clone; $F_{3,92}=3.14$,

p=0.029). Comparing only the two parasite clones which had identical AMA-1 protein sequence but differed in virulence (AS vs. CB), I found that the level of protection marginally differed, with the effect of vaccination greater against the less virulent clone (treatment*parasite; $F_{1,56}=3.7$, $p=0.057$).

During the chronic phase of the infection, the anti-parasitic effect of vaccination varied across parasite clone (Figure 3.7b: treatment*parasite; $F_{4,64}=2.7$, $p=0.0381$). Vaccination conferred protection against hosts infected with the heterologous parasite clone AS (AS vaccinated versus AS control; $t_{64}=3.2$, $p=0.0008$), but not against infections with parasite clones DK (DK vaccinated versus DK control; $t_{64}=0.2$, $p=0.4$), CB (CB vaccinated versus CB control; $t_{64}=0.08$, $p=0.46$), BC (BC vaccinated versus BC control; $t_{64}=0.07$, $p=0.47$) or AQ (AQ vaccinated versus AQ control; $t_{64}=1.3$, $p=0.9$).

Removing the homologous parasite clone DK from the analysis, the effect of vaccination varied against the heterologous parasite clones (treatment*parasite clone; $F_{3,53}=3.5$, $p=0.02$). Comparing only the two parasite clones which were identical within AMA-1 but differed in virulence (AS vs. CB), I found that the level of protection differed, with the effect of immunisation greater against the less virulent clone (treatment*parasite; $F_{1,32}=6.96$, $p=0.013$).

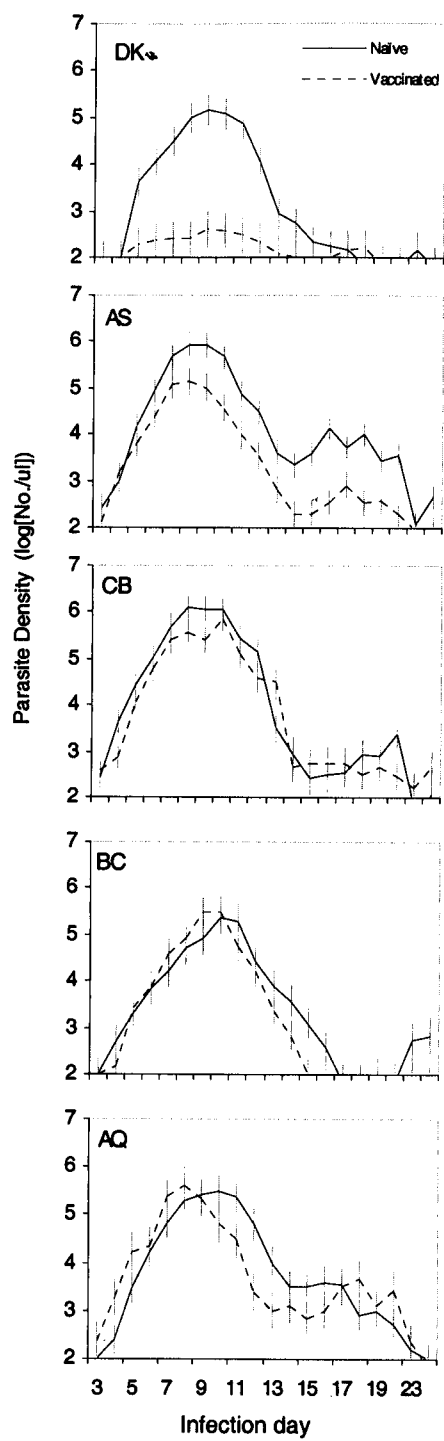


Figure 3.4. Legend on next page.

Figure 3.4. Daily parasite densities for vaccinated and naive hosts challenged with different parasite clones. The solid line represents the unvaccinated hosts, while the dashed line represents the vaccinated. Each line represents the mean of each treatment group (averaged across experimental block \pm s.e.m.) of up to 17 mice. DK is the parasite clone from which the AMA-1 vaccine was derived. Parasite clones AS, CB and BC all of which differed from the DK AMA-1 at 18 residues, followed by parasite clone AQ which differed at 30.

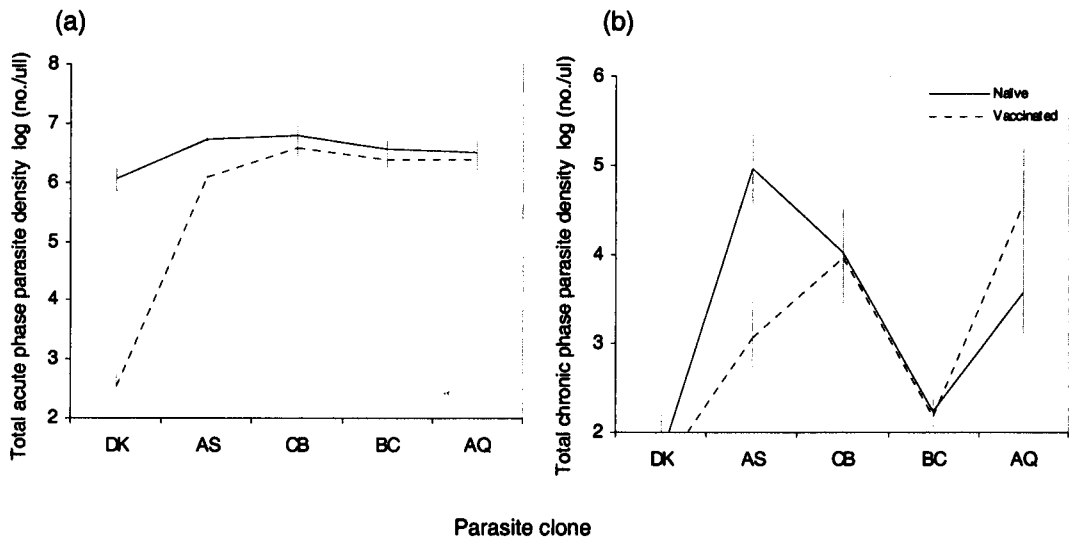


Figure 3.5. Total parasite density during the (a) acute phase and (b) the chronic phase for the five parasite clones in naïve and vaccinated hosts. The solid line represents the naïve hosts, while the dashed line represents the vaccinated. Each line is the average total density (least-squares mean \pm SE from analysis) of up to 17 mice.

3.5. Discussion

I used the rodent malaria model system of *P. chabaudi* in laboratory mice to determine the effect of parasite genetic diversity on the efficacy of a blood stage AMA-1 vaccine. To do this, I sequenced the *ama-1* gene of seven *P. chabaudi* clones and based on both their similarity to the vaccine-derived parasite and their virulence, chose a subset for experimental infections. I included three parasites of similar virulence but that differed in their genetic similarity at the vaccine target and another pair identical at the vaccine target but differing in virulence. I found that sequence diversity as well as other parasite factors were important in vaccine efficacy. These other factors may be correlated with virulence.

Parasite clones AS, BC and CB were the most similar clones to the vaccine derived parasite DK, differing at eighteen amino acid residues, while parasite clone AQ differed at thirty. I found that protection was conferred against the homologous parasite type DK and heterologous parasite AS, but not CB, BC and AQ. Comparing infections from parasite clones AS and CB infections which are identical at the AMA-1 vaccine target region but differ in virulence, I found that that vaccinated hosts infected with the less virulent parasite AS had lower parasite densities than the unvaccinated control. In contrast, parasite density did not differ between vaccinated and unvaccinated hosts infected with the more virulent clone CB (Figure 3.6a-b).

This experiment highlights the potential for anti-replication vaccines to act as agents of selection. In particular, it emphasises that factors other than diversity at the target antigen can influence vaccine efficacy. As noted above, the Combination B vaccine trial led to the selection of a more virulent variant (Genton et al. 2002). In this field trial it is not clear as to whether the FC27 type parasites were maintained in the population due to their lack of

similarity to the allele in the vaccine or some other factor such as parasite virulence. Similarly, it is not clear from my experiments if protection was conferred against parasite clone DK due to its 100% homology to the vaccine antigen or whether it arose due to the avirulent nature of the parasite clone.

One explanation for the protection observed against the avirulent parasite AS, but not the more virulent CB could be that virulent parasites are better able to be maintained in immune environments. If true, this suggests that immune hosts could be more effective at selecting for virulence than naïve hosts. Evidence for such has previously been observed by Mackinnon & Read (2004a), where clones of *P. chabaudi* were serially passaged through naïve and semi immune hosts. After passage, it was found that all lines had increase in virulence, but that this increase was greater in semi-immune hosts.

It is therefore crucial to determine if a disproportional benefit of virulence exists in vaccinated/immune environments. Using AMA-1 vaccinated hosts I am continuing this work on two fronts. In the first set of experiments, I am using serial passage to experimentally evolve the parasite clone DK. Serial passage involves the transfer of parasites from one host to another, often resulting in increased parasite virulence (Ebert 1998; Mackinnon & Read 2004a). This occurs as the parasite variant at the highest frequency in the transfer inoculum (which we assume is the most virulent) has the selective advantage. The use of an ancestral and passaged form of parasite clone DK will enable the infection of vaccinated hosts with parasites that are identical at the vaccine target and similar at all other loci but differ in virulence. To date, the serial passage of DK is continuing. A pilot study comparing the ancestral and passaged DK parasites showed that after 15 transfers there was only a marginal increase in virulence.

In the second approach I have been using a virulent and avirulent form of parasite clone AS. Both avirulent and virulent forms of the parasite clone AS are derived from a moderate clone AS (for full details see: de Roode et al. 2005b). In brief, the avirulent form of AS was derived through the drug selection of the moderate clone. The virulent parasite was then derived through the serial passage of the avirulent form. To date, I have infected vaccinated and naïve hosts with the avirulent and virulent forms of the parasite clone AS. For reasons yet to be determined, no protection against either form of the parasite clones was conferred to vaccinated hosts. Due to this surprising result, I then sequenced the AMA-1 target region for both of forms of AS parasite and found them to be identical to the sequence of the clone AS reported above, to which we find protection. As this suggests that the vaccinations may not have induced immunity, this experiment is currently being repeated with ELISA performed prior to parasite challenge to ensure an immune response has been stimulated.

To the best of my knowledge, the present and proposed experiments above would be some of the first studies explicitly aimed at testing the role of virulence in vaccine efficacy. Previous rodent and primate studies using recombinant vaccines and heterologous parasite challenge are limited to homologous challenge and/or challenge with one or two heterologous parasites (Burghaus et al. 1996; Crewther et al. 1996; Rotman et al. 1999; Sierra et al. 2003; Goschnick et al. 2004; Dutta et al. 2005). In studies such as these it is often difficult to distinguish the role of virulence and diversity at the target vaccine region, as the two factors are confounded due to experimental design.

What my data highlights is that both diversity in the target region and other parasite factors are important in vaccine efficacy. Concerns about the strain specific nature of vaccines as well as their immunogenicity have led to advocacy of a multivalent, multistage vaccine strategy

(Moore et al. 2002; Mahanty et al. 2003; Good 2005). My data supports the necessity of this but also highlights that research into the other parasite factors such as virulence may be important for the future of malaria vaccine efficacy.

Altering the immune level of a population through vaccination may have evolutionary consequences. Increasing parasite virulence is one possible consequence. This could occur in several ways, including 1) disproportionate vaccine efficacy against avirulent clones, (or the immune breakthrough of virulent clones), as suggested by the results above; 2) the reduction in the fitness costs to pathogen virulence and 3) the alteration of competitive interactions (next chapter). As we move closer to a viable vaccine, it will become increasingly important to gain an understanding into the selective effects of vaccination.

4. The impact of immunity on competition in mixed genotype infections.

4.1. Abstract

In naturally diverse *Plasmodium* populations, ecological interactions between parasites can rapidly affect the evolution of important traits. Disease intervention strategies can also alter these interactions. Malaria populations are frequently diverse and hosts may harbour either single or mixed genotype infections. In *Plasmodium chabaudi* it has been shown that that this diversity leads to competitive interactions that, in accord with much theory, competition can lead to the evolution of virulence. Here, I used *P. chabaudi* to investigate whether the outcome of this competition differs by immune environment. In particular I investigate whether host immunity, generated by either vaccination or whole parasite challenge alters 1) the success of an avirulent parasite in a mixed infection compared to in a single infection and, 2) the frequency of this avirulent clone relative to that of a virulent clone when sharing a host. I found that in one out of three experiments the immune environment altered competition, however in all cases the outcome of within-host selection did not change.

4.2. Introduction

In naturally diverse *Plasmodium* populations, ecological interactions between parasites can affect the evolution of parasite traits such as virulence. Central to this evolution is how well a parasite transmits from one host to the next host. This will be influenced by many factors, including whether a parasite ends up sharing a host and if so, with whom.

Interactions between co-infecting genotype are likely to be important in the study of malaria as *Plasmodium* infections commonly consist of more than one genotype (Babiker et al. 1999b; Smith et al. 1999; Tanner et al. 1999; Babiker et al. 2000; Bruce et al. 2000; Jafari et al. 2004). In human malaria there is indirect evidence for the presence of competition² between co-infecting genotypes (Daubersies et al. 1996; Mercereau-Puijalon 1996; Arnot 1998; Smith et al. 1999; Bruce et al. 2000), however due to ethical constraints and confounding variables direct evidence of competition is difficult to find. Theory also suggests that interactions between co-infecting genotype are likely to be important. If virulent genotypes are competitively superior, mixed infections will select for increase virulence (Levin & Pimentel 1981; Bremermann & Pickering 1983; Ewald 1983; Bonhoeffer & Nowak 1994; Nowak & May 1994; van Baalen & Sabelis 1995; Frank 1996; Mosquera & Adler 1998).

Experimental comparisons between single and mixed genotype infections are therefore necessary. The use of *Plasmodium chabaudi* in laboratory mice is one model system where this is feasible. The availability of a range of parasite clones with defined virulence phenotypes and the ability to track specific genotypes in a mixed clone infections allows the outcome of competitive interactions to be determined. To date, such studies have revealed that avirulent parasites suffer more from competition than virulent parasites and that indeed virulent parasites are competitively superior (Taylor et al. 1997; de Roode et al. 2003; de Roode et al. 2004a; de Roode et al. 2004b; de Roode et al. 2005a; de Roode et al. 2005b; Bell et al. 2006; Raberg et al. 2006). Both experimental data and theoretical predictions therefore suggest that competition in mixed genotype malaria infections select for increased virulence.

²Competition is defined as an interaction between coinfecting strains, in which the population size of one strain is depressed by the presence of the other strain. I use the term competition to include resource-based interactions, direct attack, or reductions in clonal density by immune responses triggered by coinfecting clones (apparent competition).

Any intervention strategy that alters these competitive interactions could therefore affect the evolution of virulence. *Plasmodium* intervention strategies are usually designed to act against either the replicating stages or the transmission stages of the parasite. Interventions against the replicating asexual stage, which include many potential vaccines, are aimed at easing the suffering of an individual by reducing the parasite density of the infection. If this parasite reduction coincides with a reduction in environmental space due, for example to an increase in host immunity, competitive interactions could be enhanced, selecting for more virulent genotypes. This scenario is also likely if virulent parasite clones are disproportionately better at immune breakthrough than avirulent clones and this breakthrough further suppresses the densities of the avirulent clones. In contrast, if vaccination increases the resources available for co-infecting genotypes by, for example protecting the host from anaemia and increasing available red blood cells, competition could be reduced and selection for virulence lessened. In principle, either outcome is possible.

To address these ideas empirically I undertook three experiments using *P. chabaudi*, to determine if immunisation³ altered 1) the extent of competitive suppression of the avirulent parasite and, 2) the relative frequency of the avirulent clone in mixed clone infections.

In the first experiment, hosts were vaccinated with a candidate malaria vaccine antigen, asexual blood stage antigen 1, AMA-1. In chapter 3, I determined the level of AMA-1 polymorphism across a range of *P. chabaudi* clones that differed in virulence. I found that avirulent clone AS and virulent clone CB were identical to each other at the vaccine target, but somewhat differed from the vaccine-derived antigen. Competing these clones in hosts immunised with a single AMA-1 antigen, I was thus able to determine the effect of a

³ In this thesis I use the terms immunise and immunisation to refer to the stimulation of the immune system by either vaccination with a recombinant antigen or whole parasite immunisation.

genotype-transcending immune environment. As natural immunity to malaria, as well some malaria vaccines may have a strain specific component, hosts in experiment 2 were immunised with live parasites clones known to generate strain specific immune responses. This was complemented by a third experiment where I immunised with live parasites, from clones where no strain specific immune responses have been observed. This approach allowed me to explore how different immune environments may affect competitive interactions, and how this impacts the evolution of parasite virulence.

4.3. Materials and methods

4.3.1. Parasites and hosts

Table 4.1, summarises the experimental treatments of each experiment. In experiments 1 and 2, we used two genetically distinct *P. chabaudi* clones, denoted AS (pyr1A) and CB. AS (pyr1A) was derived through pyrimethamine selection from clone AS (Walliker et al. 1975). For simplicity AS (pyr1A) will be referred to as AS from here on. Clones AS and CB were chosen for experiment 2 as in previous experiments these two clones have been shown to induce strain specific immunity against each other (Jarra & Brown 1989; Snounou et al. 1989). This means that after infection with parasite clone AS followed by drug treatment, the parasite density of a subsequent infection with AS is reduced compared to a subsequent infection with CB and visa versa. In addition, parasite clones AS and CB are identical within the AMA-1 region (Chapter 3). In experiment 3, we used the *P. chabaudi* clone AS (as above), as well as the parasite clone AJ. Clones AS and AJ were chosen as they have previously been shown to induce strain transcending immunity, such that immunisation with AS results in a comparable reduction in subsequent infections with AS and AJ, and visa versa (S.J. Cheesman, pers. comm.). All clones were derived from wild-caught thicket rats from the Central African Republic (Beale et al, 1978)

In all experiments, hosts were eight-week-old C57BL/6J inbred female mice fed on 41B maintenance diet (Harlan, UK). Their drinking water was supplemented with 0.05% para-amino benzoic acid to enhance parasite growth (Jacobs 1964) and they were kept in a 12L:12D cycle.

Table 4.1. Experimental design and sample sizes. In experiment 1, immunisation involved inoculation of recombinant AMA-1 antigen in adjuvant, with a boost four weeks later. Control groups received only adjuvant at both time points. In experiments 2 and 3, immunisation involved infection with live parasites or uninfected red cells (controls), with drug cure four days later. Mice were euthanised at predetermined levels of morbidity.

| | Treatments | Immunisation | Parasite challenge | Number of mice | Number of deaths |
|---|------------|---------------------|--------------------|----------------|------------------|
| Experiment 1 Recombinant antigen immunisation | 1 | DK antigen+adjuvant | AS | 5 | 0 |
| | 2 | DK antigen+adjuvant | CB | 6 | 3 |
| | 3 | DK antigen+adjuvant | AS+CB | 6 | 1 |
| | 4 | Adjuvant | AS | 5 | 1 |
| | 5 | Adjuvant | CB | 6 | 6 |
| | 6 | Adjuvant | AS+CB | 6 | 6 |
| Experiment 2 Live parasite immunisation | 1 | naïve | AS | 4 | 0 |
| | 2 | naïve | AS+CB | 5 | 0 |
| | 3 | AS | AS | 4 | 0 |
| | 4 | AS | AS+CB | 4 | 0 |
| | 5 | CB | AS+CB | 5 | 0 |
| Experiment 3 Live parasite immunisation | 1 | naïve | AS | 5 | 0 |
| | 2 | naïve | AS+AJ | 5 | 0 |
| | 3 | AS | AS | 5 | 0 |
| | 4 | AS | AS+AJ | 5 | 0 |
| | 5 | AJ | AS | 5 | 0 |
| | 6 | AJ | AS+AJ | 5 | 0 |
| | 7 | AS+AJ | AS | 4 | 0 |
| | 8 | AS+AJ | AS+AJ | 4 | 0 |
| Total | | | | 94 | 17 |

4.3.2. Stimulation of immune response

In experiment 1 (Table 4.1), refolded and reduced AMA-1 derived from parasite clone DK was emulsified in Montanide ISA 720 (Seppic). Mice were injected intraperitoneally with 10 ug of protein in a 100ul emulsion. Boost immunisations were conducted 4 weeks after the primary immunisation. Control mice were injected with 100ul PBS emulsified in Montanide ISA 720. This protocol was adapted from Anders et al. (1998).

In experiments 2 and 3 (Table 4.1), mice were injected a dose of 10^4 parasites of either AS, CB, AJ or AS+AJ, followed by drug treatment. The immunising dose of parasites was prepared from infected red blood cells from donor mice. Thin blood smears and red blood cell counts were used to determine the parasite densities (flow cytometry; Beckman Coulter). Blood from these donor mice was diluted in calf serum solution (50% heat inactivated calf serum, 50% ringer solution [27mM KCl, 27 mM CaCl_2 , 0.15M NaCl], 20 units of heparin per mouse). Four days after infection, all the mice were dosed orally with 20mg/kg mefloquine using a lubricated catheter. This procedure was repeated over the next two days. Previous studies have shown that this protocol results in semi-immunity (Buckling & Read 2001; Mackinnon & Read 2003, 2004a). Thin blood smears were taken to ensure that the treatment had cleared the infection. Control mice received 10^4 red blood cells from a naïve hosts, followed by mefloquine as above.

4.3.3. Experimental design and inoculation of mice with parasites.

Experiment 1 (Table 4.1): Two weeks after vaccination or sham injection, mice were separated into 3 groups and challenged with either AS alone, CB alone or a mixture of AS + CB. Mice infected with a single parasite clone received 10^5 parasites, whereas mice infected with both clones received 2×10^5 parasites made up of 10^5 of each. Although mice infected with both clones received double the dose of parasites compared to the controls, we know from previous work that a two-fold difference in parasites numbers has a negligible effect on parasite dynamics and virulence (Timms et al. 2001)

Experiment 2 (Table 4.1): Four weeks after immunisation mice were challenged with 10^6 parasites of either AS alone, CB alone or a mixture of 10^6 AS+ 10^6 CB.

Experiment 3 (Table 4.1): Four weeks after immunisation mice were challenged with 10^6 parasites of either AS alone, AJ alone or a mixture of 10^6 AS+ 10^6 AJ.

For all experiments, inoculations were prepared from donor mice by diluting blood in calf serum solution (50% heat inactivated calf serum, 50% ringer solution [27mM KCl, 27 mM CaCl₂, 0.15M NaCl], 20 units of heparin per mouse). Each mouse was injected intraperitoneally, with a volume of 0.1ml.

4.3.4. Monitoring of individual clones in mixed infections

In all experiments, individual clones in mixed infections were monitored by collecting 5 ul samples of tail blood into citrate saline. After 1 min centrifugation at 13, 000 r.p.m, we removed the supernatant and stored the pelleted blood at -70°C for subsequent DNA extraction using Instagene Matric (BioRad). We used real time quantitative PCR to measure the DNA concentration of both clones in these samples (Cheesman et al. 2003) with primers as in Bell et al. (2006). The real time protocols cannot distinguish between asexual and gametocytes and so they estimated the densities of all parasites. In the data analyses we treated these estimates as asexual density.

4.3.5. Trait definition and statistical analysis

As I am ultimately concern about the evolution of virulence, I focused my experimental design and analyses on being able to determine competitive effects on the avirulent clone. If competition is occurring, the parasite density of the avirulent clone in a mixed clone infection would be lower than the parasite density of the avirulent clone in a single infections. In all

experiments, the parasite density of the avirulent clone (clone AS) was therefore used as the response variable in the statistical analyses. Due to the 24 hour cycle of *Plasmodium chabaudi*, daily parasite counts allow the quantification of all of the parasites present. All density data was transformed using $[\log_{10} (\text{density} + 10)]$ to meet normality and homogeneity of variance assumptions. All analyses were performed as repeated measures using PROC MIXED in SAS 8.2 (SAS Institute 1999) with the repeated statement (subject = mouse) and the Satterthwaite approximation of the denominator DF. For all the models I first fitted the maximal models including and all two and three-way interactions. I then minimized the models by removing non-significant terms ($p > 0.05$), beginning with the highest-level interaction. In no cases were any of the three-way interactions significant, so we do not report them. The main effect of 'day', as well all two-way interactions with 'day' are not reported as the well known dynamic kinetics of malaria infections are not the focus of this study. Complementing all of the repeated measures analyses, we also analysed total parasite densities. Total parasite densities are calculated as the sum of the parasite densities between days 4-20. Analysis was completed using General Linear Models (JMP in 5.1).

4.3.6. Immunisation and competition

To investigate if the extent of competition was influenced by immunisation I compared the parasite density of the avirulent clone (clone AS) in single and mixed clone infections, in immunised and non-immunised environments. The effect of immunisation (immunised or not immunised), multiplicity (single or mixed clone infection) and an immunisation-by-multiplicity interaction on the parasite density of the avirulent clone was determined using repeated measures analysis. If competition is either enhanced or alleviated in the immunised hosts, there would be a significant interaction between immunisation and multiplicity.

To investigate if the extent of competition was influenced by the genotype of the clone(s) used in the live parasite immunisations, we also analysed immune animals only to determine the role of genotype specificity (immunising clone-by-multiplicity). Due to the lack of treatment group AS-alone in CB hosts (see table 4.1), we can only look at the effect of immunising clone in Experiment 3.

4.3.7. Immunisation and within-host selection

To determine if the relative frequency of the avirulent clone (AS) was affected by immunisation, we compared the relative density of AS to its competing clone, in the naïve, vaccinated and immunised animals. All proportion data was transformed using $\text{asin}(\sqrt{x})$, to meet normality and homogeneity of variance assumptions. All analyses were performed as repeated measures using PROC MIXED in SAS 8.2 (SAS Institute 1999) with the repeated statement (subject = mouse) and the Satterthwaite approximation of the denominator DF. The main effects were vaccination or immunisation, day and their interactions. To investigate if the proportion of AS was influenced by the genotype of the clone(s) used in the immunisations, we then analysed immune animals only.

4.4 Results

4.4.1. Immunisation and competition

4.4.1.1. *Experiment 1: Vaccine-induced immunity (AS and CB)*

During the experiment, 17 out of 34 mice died after their peak parasite density (Table 4.1). Of these, nine were infected with parasite clone CB, three of which were vaccinated and six naïve. Infections of AS+CB resulted in seven deaths, with one in a vaccinated host and the remaining six others in naïve. The remaining death was in a naïve host infected with parasite clone AS. This last death was highly uncharacteristic and as the mouse also had an atypically low parasite density it was removed from further analysis. High levels of mortality were observed in this experiment. We can offer no definitive reasons for these high levels apart from potential quality differences in the hosts. Due to this, mortality repeated measures analysis and total parasite density were only completed up to day 11 for this experiment.

In addition, two of the five vaccinated hosts infected with the less virulent parasite clone AS had extremely low level parasite densities (maximum of 2×10^4 parasites/ul), compared to the remaining three (maximum of 2×10^6 parasites/ul). To provide a conservative analysis, we therefore report the following results with their inclusion and exclusion.

The parasite density of clone AS in single and mixed infections, in different immune environments is shown in Figure 4.1a-c. Repeated-measures analysis including the two low level infections showed that vaccination reduced parasite densities (Figures 4.1a, b, d: vaccination: $F_{1,31.1} = 7.3$, $p=0.01$). The parasite density of the avirulent clone in single infections did not differ from the parasite density of the avirulent infection in the mixed clone infection, indicating that competition in the mixed clone infections was not occurring (multiplicity: $F_{1,29.6} = 0.22$, $p=0.64$). The multiplicity-by-vaccination interaction was also not

significant (Table 4.2). Removing the two mice with low level infections, we found that the parasite density of clone AS was reduced by both vaccination and competition and that these effects were not interacting (Table 4.2, Figure 4.1a,c,e: vaccine*day; $F_{7, 87.1} = 6.1$, $p < 0.0001$, multiplicity; $F_{1, 23} = 4.5$, $p = 0.045$). In vaccinated and naïve hosts, competition reduced the total parasite density by 44% and 40% respectively. Due to the death of naïve hosts infected with AS and CB, it was not possible to test whether competition was affected by vaccination beyond day 11. However, among vaccinated hosts only, there was no evidence of competition, when either including or excluding the low level infections (Figures 4.1b,c: $F_{1, 10.3} = 0.03$, $p = 0.85$, $F_{1, 7.9} = 0.2$, $p = 0.66$, respectively).

The parasite density of the more virulent parasite clone CB was reduced by vaccination, but not by competition such that, the parasite density of clone CB was the same in single and mixed clone infections (vaccination: $F_{7, 26.9} = 7.9$, $p = 0.009$, multiplicity; $F_{1, 26.4} = 2.3$, $p = 0.14$, vaccine*multiplicity; $F_{1, 25.4} = 1.5$, $p = 0.22$).

Thus, we found no evidence that vaccination enhanced or alleviated competition.

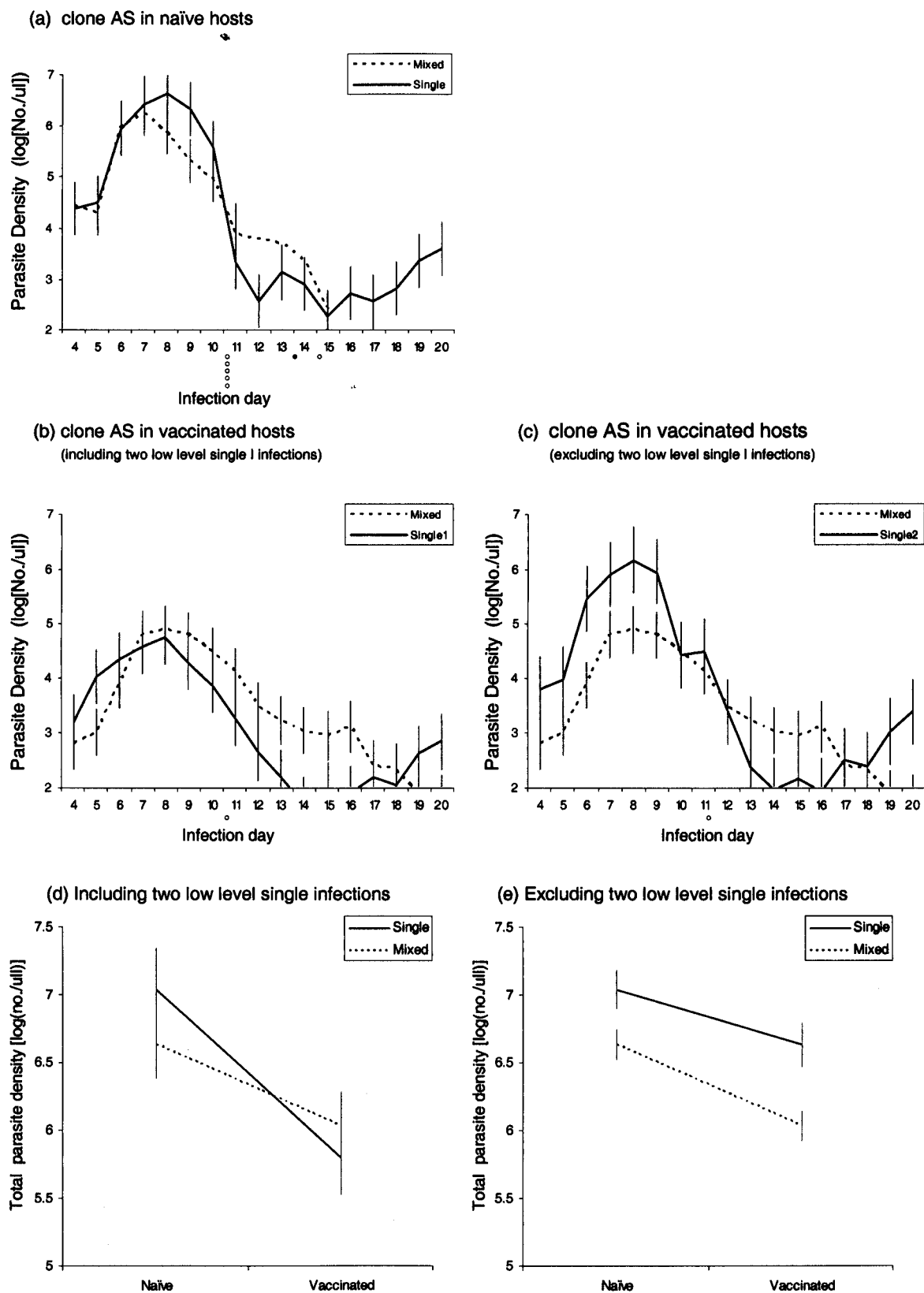


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Figure 4.1

Parasite density of parasite clone AS over time. Graphs compare the avirulent parasite clone AS in single and mixed clone infections in (a) naïve hosts (b) vaccinated hosts including two low level infections and (c) vaccinated hosts excluding the low level infections. The solid line represents clone AS when alone in the host, while the dashed line shows clone AS when in a mixed clone infection. The magnitude of competition is the difference between the solid and dashed line. Plotted points are the average parasite density (least-squares mean \pm SE from analysis) of up to 6 mice.

Total parasite density of clone AS is shown in (d) including the two low level infections and (e) excluding the two low level infections. The solid line represents the difference between clone AS when alone in naïve and vaccinated hosts. The dashed line shows the difference between clone AS when in mixed infections, in naïve and vaccinated hosts. The difference between the solid and the dashed line indicates the magnitude of competition. Plotted points are the average total parasite density (least-squares mean \pm SE from analysis) of up to 6 mice.

4.4.1.2. Experiment 2: Live parasite immunisation 2 (AS and CB).

The density of the avirulent parasite clone AS in single and mixed clone infections, in naïve and immune environments is shown in Figure 4.2a-b. Repeated-measures analysis showed that immunised hosts had lower parasite densities than naïve hosts (Table 4.2, Figures 4.2a,b,c: immunisation; $F_{1, 51.4} = 143.5$, $p < 0.0001$). The parasite density of the avirulent clone AS, did not differ between single and mixed clones infections indicating that competition in the mixed infection did not occur (Table 4.2, Figures 4.2a,b,c: multiplicity; $F_{1, 54.5} = 0.83$, $p = 0.36$).

As we were unable to detect competition during the entire infection, we examined the acute and chronic phases of the infection separately. During the acute phase, competitive suppression of clone AS was occurring, with the magnitude of this effect the same in both naïve and immunised hosts (Figure 4.2d: multiplicity; $F_{1, 36.3} = 5.4$, $p = 0.028$, multiplicity*immunity; $F_{1, 34.5} = 0.94$, $p = 0.34$, respectively). During the chronic phase, no effect of competition was observed (4.2e: multiplicity: $F_{1, 34} = 0.26$, $p = 0.6$, competition*immunity; $F_{1, 30.9} = 0.8$, $p = 0.37$, respectively)

Thus competition was neither enhanced nor alleviated by prior immunisation with live parasites.

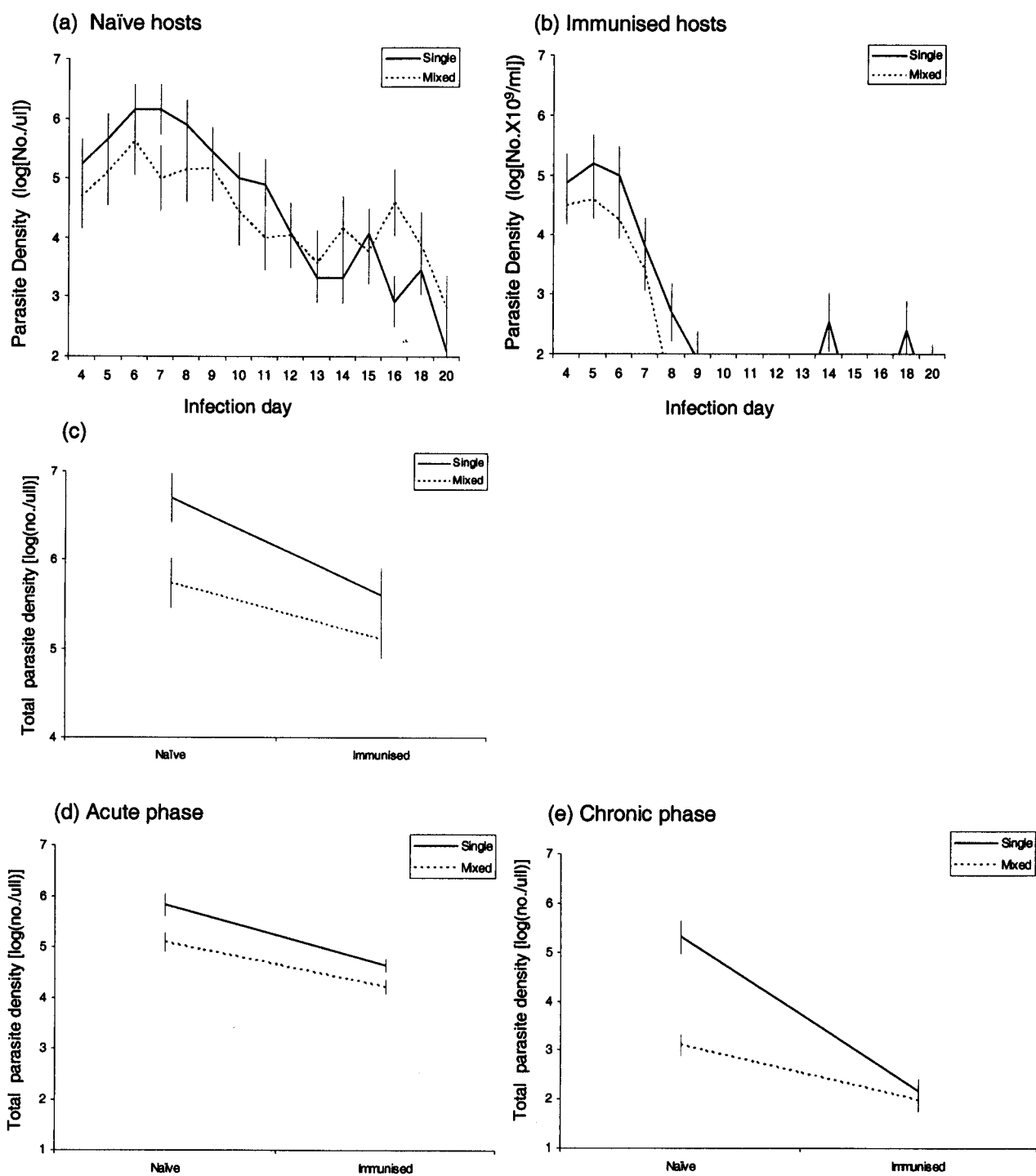


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Figure 4.2.

Parasite density of clone AS over time in (a) naïve hosts or (b) host made immune through live parasite immunisation. The solid line represents clone AS when alone in the host, while the dashed line shows clone AS when in a mixed infection. The magnitude of competition is the difference between the solid and dashed line. Plotted points are the average parasite density (least-squares mean \pm SE from analysis) of up to 5 mice.

Total parasite density of clone AS in naïve and immunised hosts, in single and mixed clone infections (c) during the entire infection, (d) during the acute phase and (e) and during the chronic phase. The solid line represents the difference between clone AS when alone in naïve and immunised hosts. The dashed line shows the difference between clone AS when in competition in naïve and immunised hosts. The difference between the solid and the dashed line indicates the magnitude of competition. Plotted points are the average total parasite density (least-squares mean \pm SE from analysis) of up to 5 mice.

4.4.1.3. Experiment 3: Live parasite immunisation 1 (AS and AJ)

Figure 4.3a and b illustrate the density of parasite clone AS in single and mixed clone infections, in naïve and immune environments. Repeated-measures analysis showed that competition and immunity were interacting, such that competition was alleviated in immunised hosts (Table 4.2). Overall, competition reduced the density of AS in naïve and immune mice by 65% and 40 % respectively (Figure 4.3c).

Considering immune hosts only, we found that the parasite density of clone AS was influenced by the presence of the coinfecting clone, but was not affected by the genotype of the immunising clone (Figure 4.4: multiplicity; $F_{1, 42.4}=5.3$, $p=0.026$, immunising clone; $F_{2, 42.4}=1.1$, $p=0.35$, respectively). The level of competition was the same in all of the immune hosts (Table 4.2).

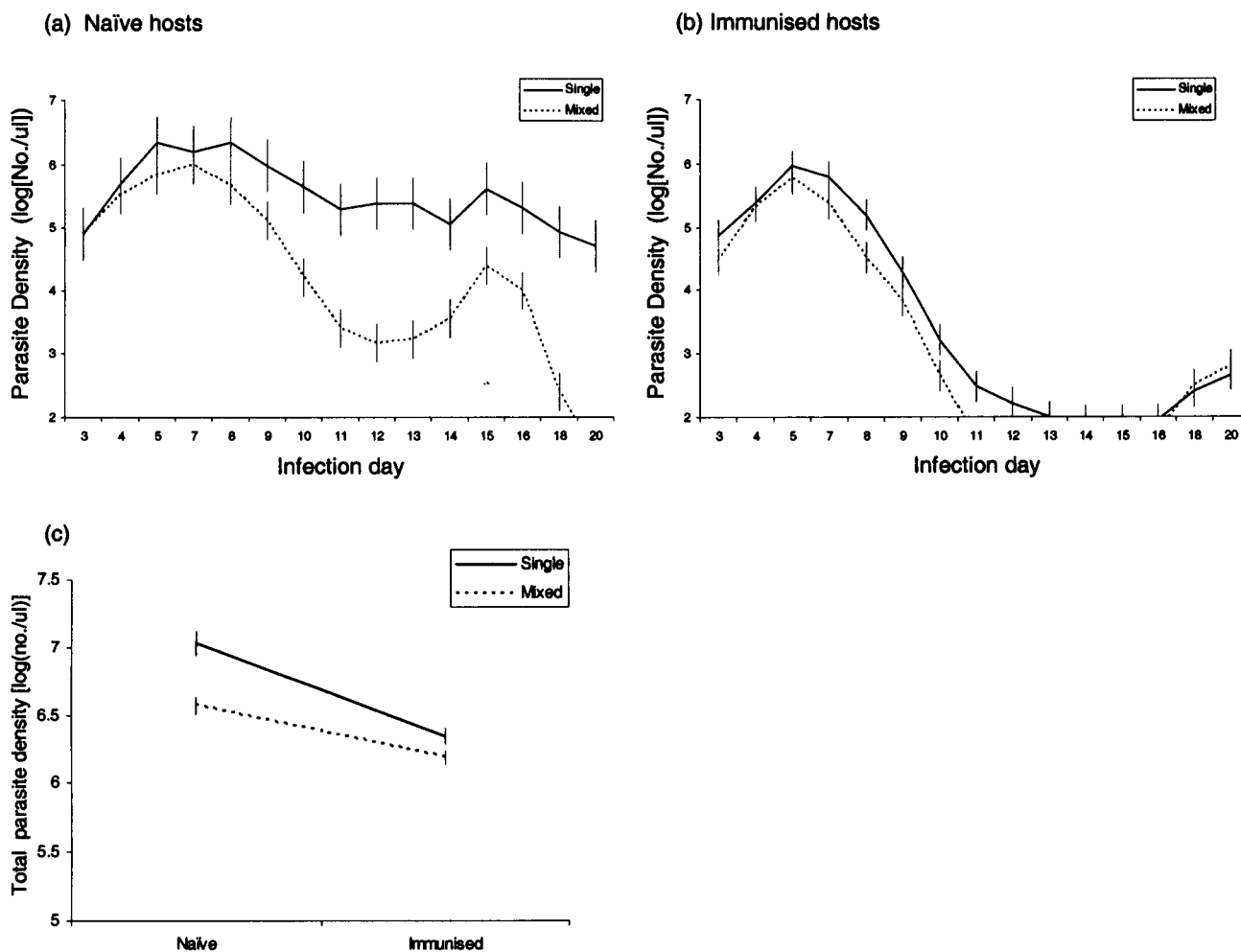


Figure 4.3. Parasite density of clone AS over time in (a) naïve hosts or (b) host made immune through live parasite immunisation. The solid line represents clone AS when alone in the host, while the dashed line shows clone AS when in a mixed infection. The magnitude of competition is the difference between the solid and dashed line. Plotted points are the average parasite density (least-squares mean \pm SE from analysis) of up to 5 mice.

The total parasite density of clone AS in naïve and immunised hosts, in single and mixed clone infections (c) The solid line represents the difference between clone AS when alone in naïve and immunised hosts. The dashed line shows the difference between clone AS when in a mixed infection, in naïve and immunised hosts. The difference between the solid and the dashed line indicates the magnitude of competition. Plotted points are the average total parasite density (least-squares mean \pm SE from analysis) of up to 5 mice.

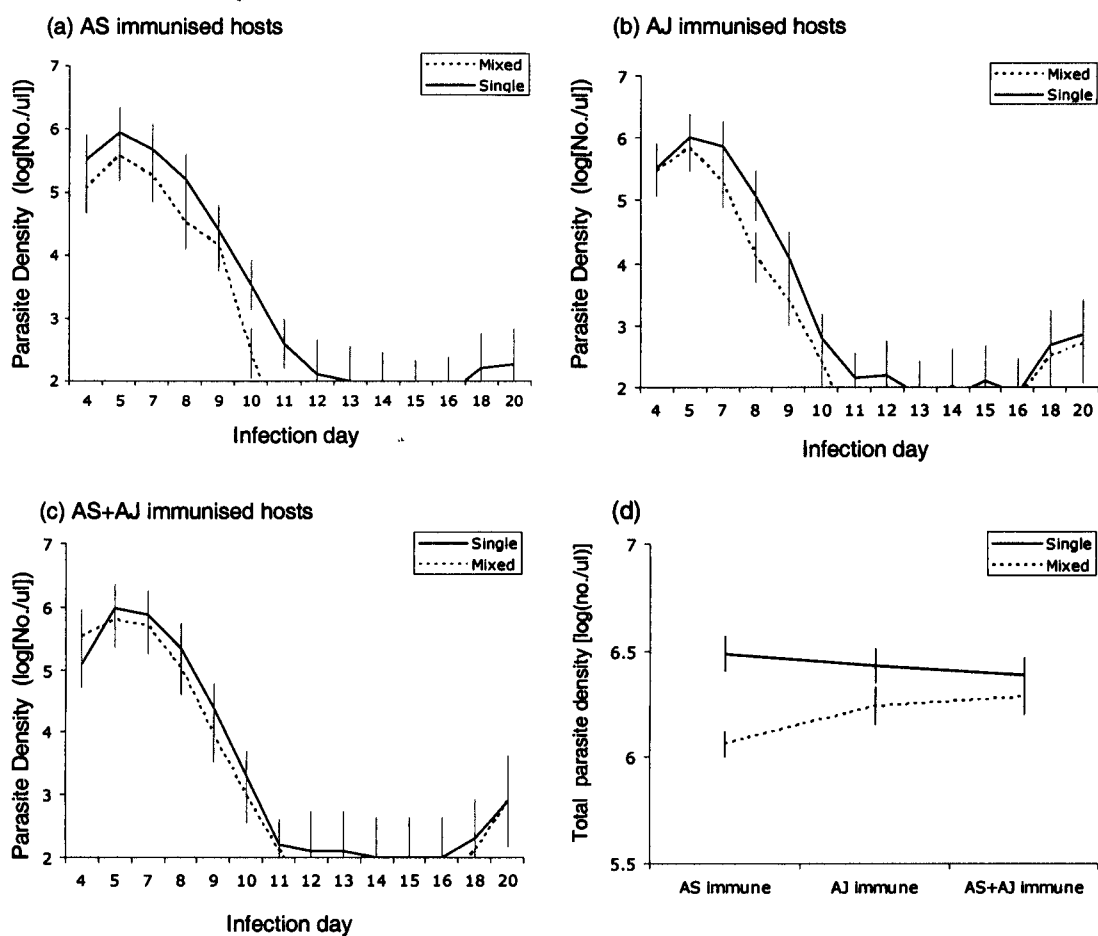


Figure 4.4 Parasite density of clone AS in hosts immunised with parasite (a) AS, (b) AJ or (c) a mixture of both. Plotted points are the average parasite density (least-squares mean \pm SE from analysis) of up to 5 mice.

Total parasite density of the hosts immunised with parasite (a) AS, (b) AJ or (c) a mixture. Plotted points are the average total parasite density (least-squares mean \pm SE from analysis) of up to 5 mice.

Table 4.2. Host immunity*competition interaction terms from repeated measures analysis and total parasite density analysis. In experiments 1 and 2, the lack of significant immunity*competitions interactions indicates that the level of competition was the same in both the immunised/vaccinated and naïve hosts. The alleviation of competition was seen in Experiment 3, with an increase of the less virulent clone AS in the immunised hosts compared to the naïve.

| | Repeated measures | Total parasite density |
|--|--|--|
| <i>Experiment 1 (AS+CB)</i> vaccinated vs. naïve, including all mice vaccinated vs. naïve, excluding 2 low level infections | $F_{1, 28.9} = 0.05, p = 0.8$ $F_{1, 21.2} = 1.1, p = 0.31$ | $F_{1, 17} = 0.56, p = 0.46$ $F_{1, 15} = 0.92, p = 0.35$ |
| <i>Experiment 2 (AS+CB)</i> Immunised vs. naïve | $F_{1, 50.8} = 0.1, p = 0.76$ | $F_{1, 19} = 0.39, p = 0.33$ |
| <i>Experiment 3 (AS+AJ)</i> Immunised vs. naïve Immunised hosts only | $F_{1, 70.5} = 6.6, p = 0.01$ $F_{2, 40.1} = 1.0, p = 0.35$ | $F_{1, 38} = 2.4, p = 0.1$ $F_{2, 22} = 1.2, p = 0.29$ |

4.4.2. Immunisation and within-host selection

To determine if the relative frequency of the avirulent clone differed by immunisation, we compared the relative densities of AS and its competing clone in the naïve and immune animals. Due the death of naïve hosts infected with AS +CB in Experiment1 and the lack of immune mice with infections during the chronic phase in Experiment 2, we did this analysis up to day 11 only (Table 4.3)

I found that in all experiments the frequency of AS in mixed infections did not differ between the vaccinated and naïve hosts (Figure 4.5, Table 4.4). Thus, there was no evidence that immunisation affected the strength of within-host selection imposed by coinfection.

Table 4.3

The number of mice that were parasite positive during the chronic phase of the infection after injection with a mixed infection.

| | | | | |
|-----------------------------|-----------------------|---------------------------------|---------------------------------|------------------------------------|
| Experiment 1 (AS+CB) | Naïve n/a | Vaccinated 2 \ 5 | | |
| Experiment 2 (AS+CB) | Naïve 5 \ 5 | AS Immunisation 0 \ 4 | CB immunisation 1 \ 5 | |
| Experiment 3 (AS+AJ) | Naïve 5 \ 5 | AS Immunisation 0 \ 5 | AJ Immunisation 3 \ 5 | AS+AJ Immunisation 2 \ 5 |

Table 4.4

The F ratio and p value for the interaction term from the analysis of the proportion of AS in vaccinated/immunised hosts versus naïve hosts.

| | Acute phase (days 4-11) | Chronic phase (days 12-20) |
|--|---|---|
| Experiment 1 (AS+CB) | $F_{1,9}=0.64, p=0.44$ | n/a |
| Experiment 2 (AS+CB) Immune vs. naïve Immune hosts only | $F_{1,8.4}=0.02, p=0.89$ $F_{1,6.4}=4.2, p=0.08$ | n/a n/a |
| Experiment 3 (AS+AJ) Immune vs. naïve Immune hosts only | $F_{1,27.5}=2.8, p=0.1$ $F_{3,26.2}=1.6, p=0.2$ | $F_{1,13.7}=0.19, p=0.67$ $F_{2,13.1}=2.6, p=0.11$ |

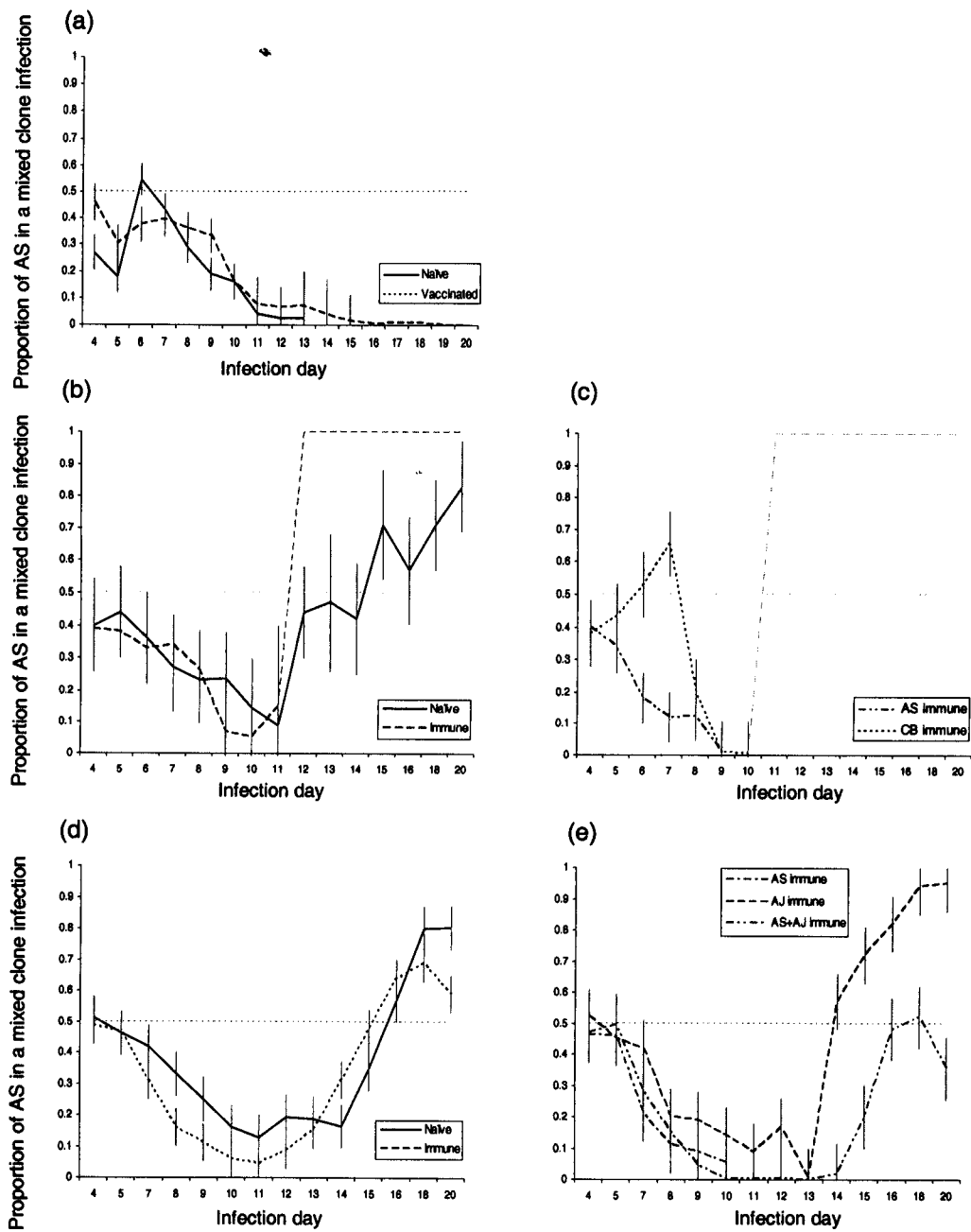


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Figure 4.5. Proportion of AS within a mixed clone infection in (a) naïve and vaccinated hosts (Experiment 1) (b) immunised and naïve hosts (Experiment 2). During the chronic phase of the infection only 1 immune host was parasite positive. This mouse had been immunised with parasite clone CB and was parasite positive for the AS clone only. This host is represented by the dashed dark grey line. (c) either AS or CB immunised hosts (Experiment 2). The dashed dark grey line represents the CB immunised host, positive for parasite clone AS only, as above. (d) immunised and naïve hosts (Experiment 3) (d) either AS immunised, AJ immunised or AS+AJ immunised hosts (Experiment 3). All hosts immunised with parasite clone AS were parasite negative for parasite clone AS after day 10. In graphs (a), (b) and (d), the solid lines represent the average proportion of clone AS when in naïve hosts, while the dashed lines shows the proportion of clone AS when in vaccinated or immunised hosts. In graphs (c) and (e), the dashed lines represent the average proportion of clone AS when in immunised hosts, by immunising clone. The horizontal grey dashed line shows the frequency of 50%. Plotted points are the average proportion of AS in a mixed infection (mean.± s.e.m.)

4.5. Discussion

In this study, I undertook three experiments to determine if immunisation altered the extent of competitive suppression and/or the relative frequency of avirulent clones in mixed infections. In the first experiment, hosts were vaccinated with a candidate malaria vaccine antigen, asexual blood stage antigen 1 (AMA-1). In experiment 2 and 3 hosts were immunised by live parasite challenge. This approach allowed me to explore the effects of a genotype transcending and genotype specific immune environments, as well as mimic recombinant vaccine induced immunity (Experiment 1) and the more natural live parasite induced immunity (Experiment 2 and 3).

In all three experiments, I found that parasite clone AS reached lower densities in mixed infections than in the single infections. Importantly, the magnitude of this competitive suppression did not *increase* in any of the immunisation treatments. This suggests that selection for increased virulence via competitive ability is not greater in immune environments. This is seen most clearly in Experiment 1, where I found that in naïve hosts, the density of avirulent clone was reduced by 40% when in a mixed infection, while in vaccinated hosts the reduction associated with the mixed infection was similar at 44%. Thus, mixed infections were costly for the avirulent parasite clone, but this cost was the same in both naïve and vaccinated hosts. By contrast, in Experiment 3 competitive alleviation was observed. The density of avirulent parasites was reduced by 65% in naive hosts, but in immune animals the competitive suppression was only 40%.

However, although immunisation appeared to reduce the negative effects of competition on the avirulent clone in Experiment 3, in no experiment did I find evidence that immunisation enhanced the relative frequency of clone AS relative to its coinfecting clone (Figure 4.5).

Specifically, when the relative success of the avirulent parasite in a mixed infection is compared to its success when alone, immunisation appeared (in one experiment) to alleviate competition. However, when the success of the avirulent parasite was directly compared to that of a co-infecting virulent parasite, the virulent parasite was the better competitor in both naïve and immunised environments. This suggests that the virulent clone also benefited from the alleviation of competitive effects caused by immunisation. This could not be tested directly because logistical constraints meant that the relevant treatment groups could not be included in the experimental design.

The extent to which clones suffer from competition is thought to be influenced by the specific nature of the immune response. During the acute phase of the infection, it is generally assumed that the immune response is largely non-specific, involving a general cellular immune response (Taylor-Robinson 1995; Li et al. 2001). If so, when the immune system is stimulated in a mixed genotype infection, all co-infecting clones will be affected. In contrast, during the chronic phase of the infection it is thought that strain specific mechanisms are involved (Jarra & Brown 1985; Phillips et al. 1997; Mota et al. 1998; Buckling & Read 2001). More similar clones will have a greater impact on each other than clones that are controlled by distinct immune responses.

Parasite clones AS and CB have been shown in previous studies to be relatively antigenically distinct (Jarra & Brown 1989; Snounou et al. 1989); immunity elicited against parasite clone AS is better able to clear an infection of parasite clone AS than CB and vice versa. In contrast, parasite clones AS and AJ are thought to be antigenically similar with immune responses directed against AS and AJ largely cross reactive (S.J. Cheesman, pers. comm.). In my experiments, parasite densities during the chronic phase of infections, where strain specific

immune responses would be expected, were dramatically reduced in immunised animals. However, tentative evidence suggests that immunisation occurred in a strain-specific manner. In experiment 2, only one out of the nine immunised hosts infected with a mixed infection was parasite positive. This host had been immunised with the parasite clone CB and was positive for the parasite clone AS; suggestive of CB specific immunity and in agreement with previous observations. In addition, the possibility exists in that in Experiment 3, strain specific immunity was observed with the combination of parasites thought not to elicit strain specific responses: AJ parasites were preferentially removed from AS+AJ infections allowing a non-significant increase of AS in these hosts (Figure 4.4 d). It is impossible to draw firm conclusions from this data; however I speculate that immune specificity could indeed play a role in competitive interactions during the chronic phase. If such specificity is elicited by vaccination, it would be likely to reduce competition and thus weaken selection for virulence.

If tentative evidence for immune mediated competition during the chronic phase exists, is there any evidence for immune mediated competition during the acute phase? In my experiments, the overall lack of a significant immunity*competition interaction suggests not. Previous experiments testing for immune mediated competition have led to contrasting results (Raberg et al. 2006 and V. Barclay et al. pers. com. 2006). Raberg et al (2006) compared the extent of competition in immunodeficient mice and immunocompetent mice. Immunodeficient mice were 'nude' mice that have a recessive mutation that blocks the development of the thymus. These mice have no mature T-cells and are unable to mount cell-mediated immune responses or form most types of antibodies. Using these immunodeficient mice, it was found that immune-mediated competitive suppression was occurring between days 10 -12, although no overall effect could be detected during the acute phase. Barclay et al (2006) used a similar approach where mice were antibody depleted of CD4⁺ T cells and single and mixed clone

infections were compared in depleted and intact animals. In two separate experiments using different combinations of competing clones, no evidence for immune mediated competition during the acute phase was found. Together with my results, all these data suggest that for the most part, the reduction in parasite density during the acute phase associated with competition is caused by mechanisms other than immunity.

Once possible mechanism mediating the parasite density during the acute phase in mixed clone infections is resource based competition. As asexual *P. chabaudi* parasites require blood glucose (Li et al. 2001) and red blood cells to maintain replication, competition for glucose and red blood cells seems feasible. Blood transfusion during and after the peak parasitaemia has been shown to prolong *P. chabaudi* infection in mice (Yap & Stevenson 1994). Competition experiments where resources are not limited will be necessary to determine if such a mechanism is important.

These data support both theory and experimental data that mixed genotype infections select for increased virulence, and shows that this process works in both naïve and immune environments. Importantly, I found no evidence that vaccination will further increase the selection for virulence which arises in mixed infections. What this series of experiments do not address is if virulent parasites are less readily cleared by vaccine induced immunity, the subject of Chapter 3. Further experiments would be valuable given the expectation that competition will affect virulence and that the direction of selection depends tightly on the mechanism of competition.

5. The effect of parental rearing conditions on offspring life history in *Anopheles stephensi*

5.1. Abstract

The environmental conditions experienced by parents are increasingly recognised to impact the success of offspring and to be of evolutionary significance. In this study, I tested for the existence of parental effects in the medically relevant vector *Anopheles stephensi*. By varying the larval parental environment and measuring key life history traits of their offspring, I found that the parental effects did not influence the time taken for offspring to emerge, offspring size or offspring survival. Parental effects were influential in determining the fecundity of their adult daughters. Daughters from parents reared in low food conditions produced larger egg clutches than daughters from parents reared in high food. Offspring reared in low food conditions took larger blood meals if their parents had also experienced a low food environment. Mosquito control methods resulting in environmental modification of larval habitat, as well as strategies involving the large scale rearing and release of genetically modified or sterile males may benefit from an increased understanding of how parent and offspring larval rearing conditions interact to alter mosquito ecology and evolution.

5.2. Introduction

The success of an individual is dependent on genetic background, environmental conditions, and interactions between these. One factor which is increasingly recognised to have a profound impact on individual success is the environmental conditions experienced by their parents (Kirkpatrick & Lande 1989; Bernardo 1996; Rossiter 1996; Mousseau & Fox 1998; Wolf et al. 1998). Whilst parental effects have been demonstrated in a wide range of taxa (e.g. Gilchrist & Huey 2001; Laurila et al. 2002; Reinhold 2002; Costa et al. 2003; Bateson et al. 2004; Fox et al. 2004; Andersen et al. 2005; Badyaev 2005; Benton et al. 2005; Jones & Widemo 2005; Sadd et al. 2005; Marshall et al. 2006; Moret 2006; Pieters & Liess 2006), it is not yet clear when parental effects are non adaptive (passive) or when they are adaptive.

Passive effects arise when the parental environment generates a constraint on offspring life history. For example, nutrient deprivation in mothers may result in less well-provisioned and hence smaller offspring. By contrast, parental effects may be adaptive if parents can perceive cues in their environment and adjust per offspring investment so as to optimise offspring fitness. Maternal effects in the freshwater crustacean *Daphnia* are a classic example. Mothers kept in poor conditions can for example alter offspring size, survival, fecundity or resistance to parasites (e.g. Lynch 1983; Mitchell & Read 2005; Pieters & Liess 2006). In this system, offspring are likely to find themselves in a similar environment to their mothers, so for mothers in poor environments it may be in their best interest to increase per offspring investment so that the few offspring they produce will have a greater chance of survival.

Anopheline mosquitoes (Diptera: Culicidae) are medically important vectors, responsible for the transmission of many diseases including malaria, filariasis and several arbovirus diseases. Mosquitoes are hemimetabolous insects whose eggs and larvae develop in freshwater

environments, eventually pupating into terrestrial airborne adults. This metamorphic life cycle, as well as the potential for female choice in oviposition site, means that mosquito larvae may experience a different environment to that of their parents. Female preference studies indicate that fecund mosquitoes choose oviposition sites based on many factors including the presence/absence of conspecific instars (which may indicate high food levels), food sources, mosquito content and the presence/absence of potential predators (Kiflawi et al. 2003; Mokany & Shine 2003; Bond et al. 2005; Huang et al. 2005; Pates & Curtis 2005). Such behaviour indicates that female mosquitoes have the ability to alter their offspring fitness through behaviour and raises the question of whether *anopheles* parents may alter investment in their offspring as well.

Despite the hope for large scale rearing and release of sterile males and genetically modified mosquitoes for malaria control, the effects of *Anopheles* larval parental rearing experience on offspring success are to the best of my knowledge unknown. Yet, central to the success of any breeding and release strategy is the production of large numbers of mosquitoes that have a fitness (which includes mating success) close to the wild population (Phillips 2001; Atkinson & Michel 2002; Moreira et al. 2002; Benedict & Robinson 2003; Andreasen & Curtis 2005; Ferguson et al. 2005; Helinski et al. 2006).

To determine the extent of non-behavioural parental effects on *Anopheles* fitness, I conducted a fully factored cross experiment where anopheles larvae experienced either the same or different rearing conditions to that experienced by their parents. After varying the parental environment, the offspring fitness components of emergence time, size, survival, blood meal size and fecundity were measured. As genetic and phenotypic variance is often greater in

stressful environments, particular attention is paid to parental effects in offspring reared in low food environments (reviewed in Hoffmann & Merila 1999; Danks 2006)

5.3. Materials and methods

5.3.1. Experimental design

Mosquitoes originated from a long-standing laboratory stock of *Anopheles stephensi* and were reared under standard laboratory conditions at 27 ± 2 °C, 70% humidity and in a 12: 12 light: dark cycle. Eggs were hatched in 3 plastic trays (25cm x 25cm) filled with 1.5L of distilled water. Two days after hatching, larvae from the three trays were mixed and 200 were randomly transferred to 30 ml vials containing 5 mls of distilled water, where they were reared individually (Figure 5.1a). Half of these larvae were then randomly allocated to the low food treatment group (1mg of Tetrafin food per day) and half to the high food treatment group (10 mg of Tetrafin food per day). When individuals pupated, their vial was covered with fine nylon gauze until emergence. Appropriate food levels were determined from an earlier pilot study and include the range where 100% emergence occurred. The position of the vials within the insectary was fully randomised at this stage and in subsequent stages.

On emergence, the mosquitoes were pooled into three mesh cages (30 x 30 x 30 cm) per treatments groups and provided with 10% glucose solution *ad libitum* (Figure 5.1b). Approximately one week after emergence, one anaesthetised mouse was placed on each cage from which the mosquitoes were allowed to feed for 20 minutes. One day later egg bowls were introduced to the cage for oviposition.

These eggs were then pooled and hatched in 6 plastic trays for each treatment and 400 individual larvae (200 from each parental treatment group) randomly transferred to a standard

vials as above (Figure 5.1c). The larvae were allocated to either the high or low food treatment groups (as above) and monitored daily for pupation. When individuals pupated, they were transferred to a vial that was covered with fine nylon gauze and maintained without food for survival analysis (Figure 5.1d). On death, mosquitoes were transferred to a 1 ml tube and refrigerated until the end of the experiment. At the end of the experiment the wings of each mosquito were dissected and measured from the distal to dorsal points using microscopy.

This experiment was repeated eight weeks later. In the repeated experiment, offspring were separated into two groups for either survival analysis as in the previous experiment, or for fecundity analysis after a blood meal (Figure 5.1d and e). Fecundity analysis involved transferring both male and female offspring into mating cages (30 x 30 x 30 cm), with 4 mating cages per offspring group. Five days after emergence, anaesthetised mice were placed on each of the 16 mating cages and the mosquitoes allowed feed for 20 minutes. These mice had all been infected 14 days before the feed with *Plasmodium chabaudi*, as the original intention was to determine whether vectorial capacity was affected by parental environmental conditions. However most unexpectedly on the day of the blood-meal, neither asexual nor sexual parasites were present in any of the mice and on dissection of the female mosquitoes after egg-laying, no transmission had occurred. As infected mice, were parasite negative on the day of the feed and were randomly assigned treatment groups which were replicated, any treatment effect should be unrelated to any effect of infection. Immediately after the feed, each female mosquito was transferred to a clean vial covered with fine nylon gauze for 3 days to allow all haematin (a by-product of decomposition of haemoglobin) to be excreted (Figure 5.1e), from which blood meal size was estimated (Briegleb 1980). Excreted haematin collected in the bottom of the vial was dissolved in 1 ml of 1% LiCO₃ solution. The absorbance of the resulting mixture was read at 387nm in a spectrophotometer using LiCO₃ solution as a blank and

compared with a standard curve made with porcine serum haematin (Sigma Aldrich). Solutions that were within the error range of the LiCO_3 blanks (absorbance < 0.01) were eliminated from the analysis and classified as non-feeders. After the 3-day haematin collection period, mosquitoes were moved to new 30ml tubes containing 3mls of water to allow oviposition (Figure 5.1f).

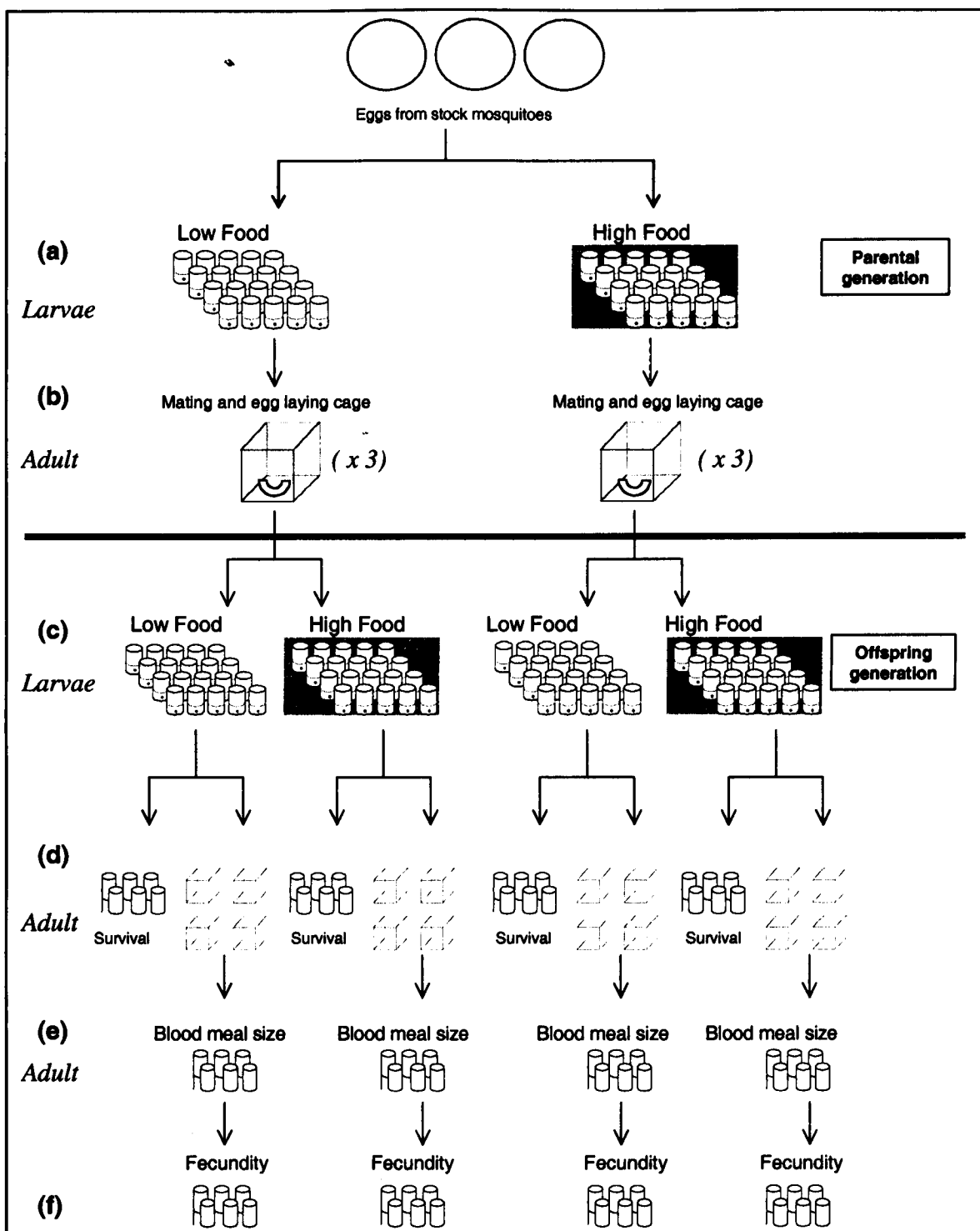


Figure 5.1. Legend on next page.

Figure 5.1.

The experimental set up. (a) In experiments 1 and 2, a total of 320 larvae were separated into the parental treatments of high and low food, with individual larvae placed in a standard 30 ml vial containing 5 mls of distilled water. (b) After emergence, adult parents were transferred to a mating cage and kept in good conditions. (c) Eggs from within treatment matings were split into high and low food. (d) In experiment 1, post emergence, the offspring were starved and their survival determined. In experiment 2, half of each offspring treatment was starve, while the other half was transferred to one of four mating cages for a blood meal. (e) After the blood meal individual females were transferred to standard vials for haematin collection. (f) After haematin collection individual females were transferred to standard vials for egg laying.

5.3.2 Trait definition

The fitness components of emergence time, survival, adult size, blood meal size and fecundity were measured. Both emergence time and survival were measured as the number of days required for either a) the larvae to emerge as an adult, post hatching, or b) taken to die post emergence. As an indicator of size, the length of one wing per mosquito from the distal to dorsal points using microscopy was measured. Haematin mass was used as an indicator of blood meal size, while fecundity was determined by counting the number of eggs laid over the 3 days following the blood meal.

5.3.3 Statistical analysis

The life history traits of emergence time and survival were analysed using Proportional Hazards (JMP in 5.1). Adult size, blood meal size and fecundity were analysed using General Linear Models. The explanatory variables were parental food (two levels), offspring food (two levels), gender (two levels) and where relevant, experimental block (two levels). For all models, a maximal model including all two and three-way interactions was fitted first. Models were then minimized by removing non-significant terms beginning with the highest-level interaction. In no cases were any of the three-way block*parent*offspring condition interactions significant and these are therefore not reported. Significant block*parent or block*offspring interactions did occur, but as they only reflected differences in magnitude between blocks, they are not reported. For the blood meal analysis as well as the fecundity analysis, the average blood meal size per replicate cage and the average number of eggs per replicate cage were used as response variables (n=16), with average adult size as a covariate. This is a conservative approach to deal with the issue of pseudo replication of treatments arising from mosquitoes fed on the same mouse. I also tested for a main effect of mouse and the interaction between mouse and the covariates (adult size and blood meal size). For both of

the response variables of blood meal size and fecundity, the main effect of mouse and the interactions were non-significant.

5.4. Results

5.4.1. Sample sizes

In experiment 1, 200 larvae were split into either a high food group or low food group, to form the parental generation. Once adults, these parents were mated within groups (three per treatment) and 400 eggs from the parental high food generation and 400 eggs from the parental low food generation were used for the offspring experiments. Of these, complete records of the emergence time, survival, gender and size were noted for 460 mosquitoes which were included in the analysis. In the repeat experiment, 120 larvae formed the parental generation, from which, 400 eggs from the parental high food generation and 400 eggs from the parental low food generation were used for the offspring analysis. Of these, emergence time was noted for 478 mosquitoes, with 140 of used in the survival trial and 287 used for blood meal and fecundity analysis. Altogether, 149 females were included the fecundity trials in which 19,844 eggs were counted.

5.4.2. Time taken to emerge

The time taken for larvae to emerge as adults post-hatching was determined by offspring food conditions and to a lesser degree offspring gender (Figure 5.2a, table 5.2-1). Larvae reared in high food conditions emerged from pupation up to 4 days earlier than larvae reared in low food conditions and males emerged on average, one day before females. The food levels experienced by the parental generation did not influence emergence time and this lack of parental influence was constant across offspring food levels (Figure 5.2a, table 5.2-1).

5.4.3. Size

Offspring larval food level was also the main factor influencing offspring adult size (Fig 5.2b, table 5.2-2). Larvae emerging from high food conditions were on average 16% larger than those emerging from low food conditions. Gender was also an important determinant of offspring size, with females being 7% larger than males (Figure 5.2b, table 5.2-2). The larval food levels of the parental generation did not influence offspring adult size (Figure 5.2b, table 5.2-2).

5.4.4. Survival

The food level experienced by the offspring was again the only factor found to be influencing adult survival (Figure 5.2c, table 5.2-3a). As expected, offspring reared in high food conditions survived for longer than offspring reared in low food conditions. Survival did not differ between the males and the females and was unaffected by parental rearing conditions (Figure 5.2c, table 5.2-3a). Larger offspring survive longer than smaller offspring ($\chi^2 = 148$, $df = 1$, $p < 0.0001$), but offspring food level was still a major determinant of survival even when controlling for offspring size (Figure 5.2c, table 5.2-3c).

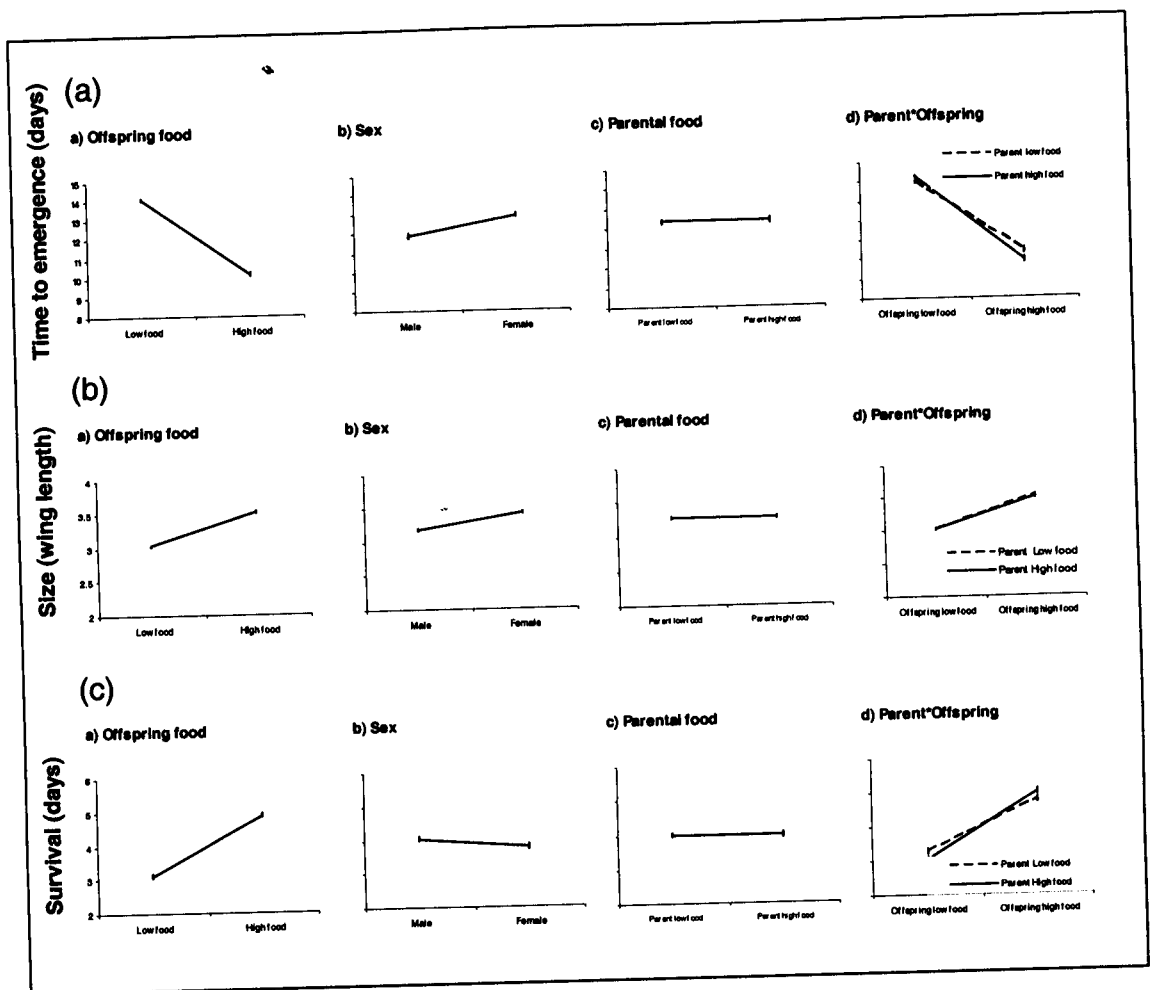


Figure 5.2. Legend on next page

Figure 5.2

5.2a: Summary graphs showing the main effect of a) Offspring food (low and high) b) Gender (male and female) c) Parental food (low and high) and the d) interaction between parent and offspring for the fitness components of emergence time. Emergence time was measured as the number of days taken post hatching to emerge as an adult. In total the emergence time of 938 mosquitoes was recorded. For this and all following analysis, 'block' interactions were fitted. Each graph including those below, represents the least square means and the associated standard error. Note that in some cases the dashed line is obscured by the solid line in the interaction graphs.

5.2b: Summary graphs showing the main effect of a) Offspring food b) Gender c) Parental food and the d) interaction between parent and offspring for the fitness components of adult size. Adult size was determined from the wing length of each mosquito. In total the wing length of 747 mosquitoes was recorded.

5.2c: Summary graphs showing the main effect of a) Offspring food b) Gender c) Parental food, and the d) interaction between parent and offspring for the fitness components of adult survival. Adult survival was determined as the number of days the mosquito remained alive, post hatching in the absence of water or glucose. In total the wing length of 747 mosquitoes was recorded.

5.4.5 Blood feeding

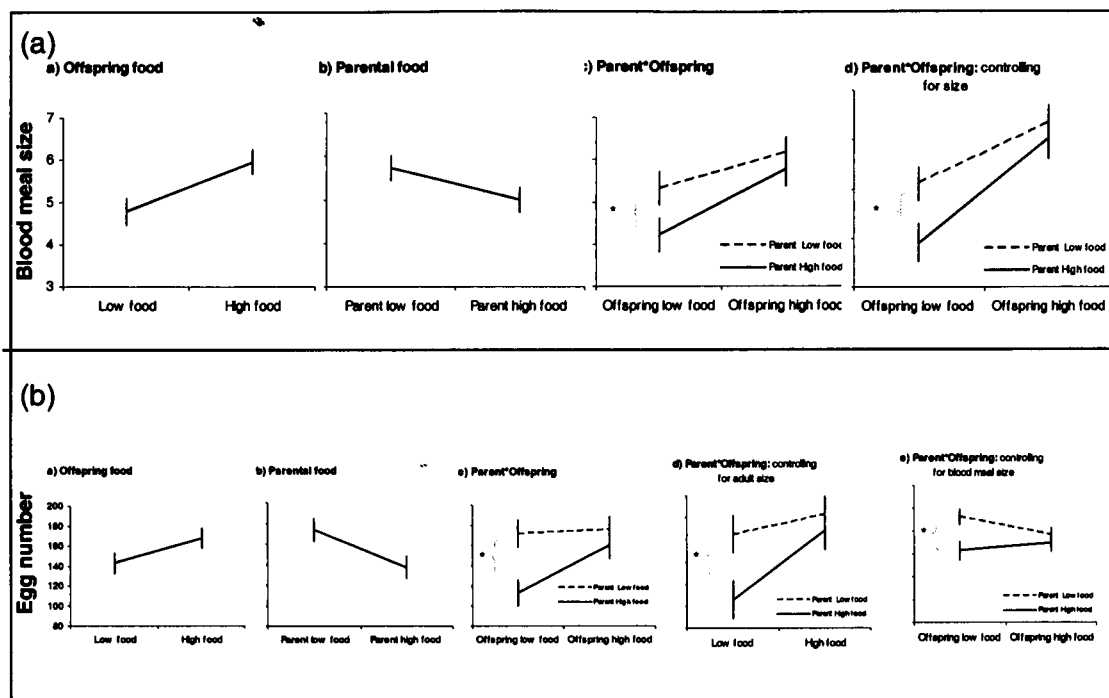
Nineteen out of the 149 females that were given access to a blood meal were classified as non feeders. Ten of these were parent low-offspring high, four were parental high – offspring low, another four were parental low – offspring low and the one remaining female was parental high – offspring high. No effect of parental food level, offspring food level or an interaction between the two influenced female propensity to feed (parent: $\chi^2 = 2.8$, $df = 1$, $p=0.09$; offspring: $\chi^2 = 2.7$, $df = 1$, $p=0.1$; parent*offspring: $\chi^2 = 2.5$, $df = 1$, $p=0.1$, respectively).

Of the 130 females that did feed, only their larval food level influenced blood meal size (Figure 5.3a, table 5.2-4a). Daughters reared in high food conditions took 25% larger blood meals than the daughters reared at low food conditions. Controlling for adult size, it was again found that blood-meal size was influenced only by the food level experience of the offspring (Figure 5.3a, table 5.2-4b). However, parental effects were apparent when the offspring reared on low food only were considered: blood meal size was influenced by the larval food level of parents, even when controlled for adult size (parent: $F_{1,6}=27.4$, $p=0.002$, parent controlled for size: $F_{1,5}=138.3$, $p<0.0001$, respectively).

5.4.6 Egg number

Parental effects influenced offspring fecundity. Offspring of parents reared in low food conditions produced more eggs than the offspring from parents reared in high food conditions (Figure 5.3b, table 5.2-5a). Parental food level influenced offspring egg number, even when controlling for offspring size (Figure 5.3b, table 5.2-5b). Considering the offspring reared on low food only, I found that the fecundity of low food daughters was influenced by the larval food level of their parents, even when controlled for adult size ($F_{1,6}=13.6$, $p=0.01$, $F_{1,5}=12.3$, $p=0.017$, respectively).

Blood meal size and egg number were positively correlated, with larger blood meal sizes resulting in increased egg production ($F_{1, 14}=34.1$, $p<0.0001$). To determine the number of eggs produced for a given blood meal size, I controlled for meal size and found that the offspring as well as parental larval food level was influencing offspring fecundity (Figure 5.3b, table 5.2-5c). Parental effects were only influencing the daughters reared at low food conditions and not daughters reared at high food ($F_{1, 5}=16.1$, $p=0.01$, $F_{1, 5}=4.5$, $p=0.09$, respectively). For the same size blood meal, the daughters reared at low food conditions were 20% more fecund if their parents had also been reared at low food levels.



* Significant difference

Figure 5.3

5.3a: Summary graphs showing the main effects of a) Offspring food and b) Parental food, as well as c) the interaction between parent and offspring and d) the interaction between parent and offspring controlling for adult size, for the fitness component of blood meal size. Haematin concentration was used as an indicator of blood meal size. For each treatment 10-15 female mosquitoes fed on 4 replicate mice. The plotted points are therefore the average blood meal size of each mosquito per mouse (n=16) and the associated standard error.

5.3b: Summary graphs showing the main effects of a) Offspring food and b) Parental food, as well as c) the interaction between parent and offspring and d) the interaction between parent and offspring controlling for blood meal size, for the fitness component of fecundity. Fecundity was determined by counting the number of eggs laid over the 3 days following a blood meal. For each treatment 10-15 female mosquitoes fed on 4 replicate mice. The plotted points are therefore the average total number of eggs laid per mosquito per mouse (n=16) and the associated standard error.

Table 5.1. The effects of parent food level, offspring food level and an interaction between them on the fitness components of emergence time, adult size, survival, blood meal size and fecundity. * = < 0.05, ** = <0.01, *** = < 0.001, **** = <0.0001

| | Fitness component | Effects | Test Statistic | p | Significance |
|----|---|--|---|--|--------------|
| 1 | Emergence time (days) | Parental food Offspring food Sex Parental*Offspring | $\eta^2 = 2.7$ $\eta^2 = 699.7$ $\eta^2 = 41.4$ $\eta^2 = 1.2$ | 0.09 <0.0001 <0.0001 0.21 | *** *** |
| 2 | Size (wing length) | Parental food Offspring food Sex Parental*Offspring | $F_{1,593} = 1.2$ $F_{1,594} = 2567$ $F_{1,594} = 900$ $F_{1,592} = 0.7$ | 0.27 <0.0001 <0.0001 0.39 | *** *** |
| 3a | Survival (days) | Parental food Offspring food Sex Parental*Offspring | $\eta^2 = 1.1$ $\eta^2 = 96.7$ $\eta^2 = 1.9$ $\eta^2 = 1.1$ | 0.30 <0.0001 0.17 0.30 | *** |
| 3b | controlling for adult size | Parental food Offspring food Sex Parental*Offspring | $\eta^2 = 0.8$ $\eta^2 = 62.9$ $\eta^2 = 1.6$ $\eta^2 = 1.6$ | 0.38 <0.0001 0.20 0.20 | *** |
| 4a | Blood meal size (haematin) | Parental food Offspring food Parental*Offspring | $F_{1,13} = 3.29$ $F_{1,14} = 7.3$ $F_{1,12} = 0.8$ | 0.09 0.017 0.39 | * |
| 4b | controlling for adult size | Parental food Offspring food Parental*Offspring | $F_{1,12} = 3.1$ $F_{1,13} = 6.9$ $F_{1,11} = 0.9$ | 0.10 0.02 0.35 | * |
| 5a | Fecundity (egg number) | Parental food Offspring food Parental*Offspring | $F_{1,14} = 6.7$ $F_{1,13} = 3.2$ $F_{1,12} = 2.5$ | 0.02 0.096 0.13 | * |
| 5b | controlling for bloodmeal size | Parental food Offspring food Parental*Offspring | $F_{1,13} = 5.9$ $F_{1,12} = 3.0$ $F_{1,11} = 2.4$ | 0.03 0.1 0.14 | * |
| 5c | controlling for adult size | Parental food Offspring food Parental*Offspring | $F_{1,11} = 16.7$ $F_{1,11} = 8.0$ $F_{1,11} = 5.6$ | 0.0018 0.016 0.037 | ** * * |
| 5d | controlling for daughters bloodmeal size and adult size | Parental food Offspring food Parental*Offspring | $F_{1,10} = 15.3$ $F_{1,10} = 7.3$ $F_{1,10} = 5.2$ | 0.0029 0.022 0.046 | ** * * |

5.5. Discussion

This study sought to establish the role of parental effects on components of *Anopheles* fitness. Key life history traits of emergence time, size, survival, blood meal size and fecundity were measured. Parental environment had variable effects on mosquito fitness. Parental effects did not influence the time taken for offspring to emerge, offspring size or survival. These traits were heavily influenced by current offspring food levels only. There was a parental effect on blood meal size, but this was only apparent in offspring reared in the low food environment. This is consistent with previous studies where maternal effects were only identified in stressful conditions (reviewed in Hoffmann & Merila 1999; Danks 2006). In my experiments, daughters raised in the low food environment took larger blood meals if their parents had also experienced low food than if their parents had experienced high food. Parental effects were influential in determining the fecundity of their daughters. Daughters from parents reared in low food conditions produced more eggs than daughters from parents reared in high food.

This effect of increased fecundity may have arisen as a facultative life history shift to compensate for decreased longevity. A reduced adult lifespan of even a few days could dramatically decrease total lifetime fecundity. In an environment where numerous bouts of reproduction are not probable, the optimal strategy may be to shift resources into one or a few larger reproductive efforts (Stearns 1989). Measuring the survival of egg laying mothers, lifetime reproductive success and egg quality would greatly aid the differentiation between adaptive and passive effects. A key question to answer would be, does increased fecundity come at the cost of reduced offspring or parental survival?

Does the potential for adaptive parental effects exist in this system? For this to be the case (i) environmental variation would need to have the potential to reduce offspring fitness (ii)

parents would need be able to predict their offspring's environment through reliable cues and (iii) parents would be able to adaptively adjust the phenotype of their offspring to the anticipated environment (Moret 2006). Evidence for environmental variation influencing mosquito fitness is abundant. Temperature, humidity, parasitism, sugar feeding and plant extracts have all been found to influence fecundity (e.g. Jahan & Hurd 1997; Jeyabalan et al. 2003; Mostowy & Foster 2004; Tripathi et al. 2004; Afrane et al. 2006). The potential for prediction and life history adjustment are less clear. Oviposition preference indicates that mothers can assess the environment their larval offspring will inhabit. However, it may be quiet a bit harder for a female mosquito to predict the environment that her offspring will inhabit, and I know of no direct evidence of such. There is much potential for work on adaptive parental effects in *Anopheles*.

Whether the parental effects I report here are passive or adaptive, they have implications for the success of disease control strategies. The ecological manipulation of larval habitats, as well as the possible large scale release of captive raised genetically modified or sterile *Anopheles*, makes the study of such effects relevant to future disease control. In the rearing and release of hatchery salmon, it has been suggested that selection for increased fecundity has resulted in decreased egg size which they linked to decreased survival (Heath et al. 2003). In the present study I have also shown parental effects on mosquito fecundity measures and although assigning adaptive significance to this result is difficult, there is no doubt that rearing conditions impacted a key life history trait. By its nature, captive breeding selects for quantity over quality. If one lesson can be learned, it should be that large rearing in benign environments may lead to their maladaptation to wild conditions.

6. General discussion

Full discussions on specific findings have been provided within each chapter. Here I briefly summarise my main findings, and then highlight general themes and future directions.

6.1. Findings

In this thesis I investigated selective pressures that could shape the ecology and evolution of malaria and its *Anopheles* host. This is dependant on the intricate relationship between the *Plasmodium* parasite, the human host and the *Anopheles* vector. Of particular interest were the effects of disease interventions on these relationships.

In chapter 2, I explored the applicability of virulence trade-off theory to the rodent malaria model system *P. chabaudi*: chapter 2 therefore forms the foundation for the rest of my thesis. In virulence trade-off theory, virulence is an unavoidable consequence of a parasite's effort to optimise its fitness, arising if parasites require extensive host exploitation to obtain transmission to the next host (Anderson & May 1982; Sasaki & Iwasa 1991; Antia et al. 1994; Ebert & Herre 1996; Read et al. 2002). An assumption of this theory is that virulence is a parasite trait which is stable across host strain. Considering four host strains and four genetically distinct parasite clones I found that virulence was indeed largely stable across all host strains tested.

I next explored whether the candidate malaria vaccine AMA-1 varied in efficacy against a range of *P. chabaudi* clones and whether vaccination affects competitive interactions between parasites. Malaria vaccines are being designed to target different stages of the parasite life cycle. Anti replication vaccines, such as the one tested, target the stage of the life cycle associated with morbidity and mortality. Theory suggests that anti replication vaccines may

increase selection for parasite virulence. I found that diversity in the target antigen region as well as other parasite factors were important in vaccine efficacy. This highlights the potential for anti-replication vaccines to select against malaria parasites. Although mixed infections in both naïve and immune environments selected for increased virulence, vaccination did not enhance or relieve this selective pressure.

Lastly, in chapter 5, I explored selection on the malaria parasite vector. Central for malaria evolution is transmission to its vector. As *Plasmodium* evolution is intricately linked to vector population dynamics, I explored environmental conditions that may influence *Anopheles* fitness. Specifically, I investigated whether the larval food level *Anopheles* parents had experienced, influenced the success of their offspring. I found that the fitness components of emergence time, adult size and survival were influenced by offspring food levels. In contrast, fecundity, and to a lesser degree, blood meal size, were influenced by parental food level. This is to the best of my knowledge the first experimental evidence for transgenerational effects in *Anopheles* fitness.

6.2 General Themes: The use of animal models

The use of animal models assumes that certain key generalities exist. Consequently, animal models have traditionally been a part of malaria research, and indeed have aided much of what we know about the biology of malaria parasites. However, the ability to culture the human malaria parasites *in vitro*, the availability of a number of *Plasmodium* genomes and differences between human and *in vivo* model infections, raises questions as to whether there are still benefits of using *in vivo* model systems.

Plasmodium chabaudi in laboratory mice is one such *in vivo* system, where in addition to its distance from the human malarias, the host and parasite are not a natural combination. Typically differences between natural malaria infections and non-natural host parasite system are numerous. For example, natural malaria infections do not often result in death, are thought to be chronic and usually result in infections of low parasite density (reviewed in Landau & Chabaud 1994) In contrast, non-natural malaria infections are usually short-lived, reach high parasite densities and often result in host death (Stevenson et al. 1982).

And yet, the rodent model systems also share many feature with human malaria. For example:

- The basic biology of rodent and human parasites are similar. In particular, *Plasmodium chabaudi* and *Plasmodium falciparum* both undergo synchronous schizogony, capillary sequestration and have a preference for mature red blood cells (Mons & Sinden 1990).
- Genome size, the number of chromosomes and the G+C content are similar between rodent and human parasites (Carlton et al. 2002; Gardner et al. 2002; Hall et al. 2005)
- Surface proteins are conserved between rodent and human parasites (Marshall et al. 1989; Waters et al. 1990; Thompson et al. 2001; Janssen et al. 2002).
- The molecular basis of drug-sensitivity and resistance show are similar rodent and human parasites (Carlton et al. 1998; Carlton et al. 2001; Walliker 2005).

In addition, the rodent malaria system enables *in vivo* investigations of parasite-host interactions in well characterised host genetic backgrounds with a variety of parasite types. In particular, the *P. chabaudi* system is the only malaria model with a range of genetically

distinct parasite clones with distinct virulence phenotypes (Mackinnon & Read 1999a; Ferguson & Read 2002).

Enhancing the rodent-malaria model system will ensure its applicability in the future. One exciting way forward would be to reconstruct mice with human immune systems or genetically modify immune-intact mice to elicit human responses (Druilhe et al. 2002; de Koning-Ward et al. 2003; Druilhe et al. 2005). One can image that these animals would be better predictors of immunogenicity and would be useful in vaccine efficacy and safety studies. With further advancement, this technology may even lead to the production of mice able to maintain human malaria parasites (Cooke & Coppel 2004).

Above all, I see rodent malaria systems as a necessity for the study of virulence evolution. Informative manipulations, such as those performed for this thesis, require experimental, whole-organism infections to gain insight into the evolution of harmful parasites. Moreover, the experiments required for the study of virulence in vertebrate hosts involve the comparison of morbidity and the determination of parasite fitness, which for ethical reasons can not be done in humans.

P. chabaudi in laboratory mice is therefore an ideal virulence model for any microparasite where virulence is a parasite encoded trait and a virulence-transmission trade-off exists. An added potential advantage of this system is that the non-natural host-parasite combination may reveal more parasite genetic variation in the novel host environment; making it possible to study virulence phenotypes that selection in natural systems would have otherwise removed. This potentially allows the unique opportunity to study both evolutionary progression and outcomes, as opposed to just the evolutionary outcomes evident in equilibrium systems.

6.3. Future directions

1) *Vaccines*. One of the major caveats of this thesis is that it is based on a limited number of parasite clones and only one candidate vaccine antigen. Further study using different parasite clones and different candidate vaccine antigens are an obvious and desirable future direction. The merozoite surface protein 1 (MSP-1) is one such antigen, thought to be largely conserved in *P. falciparum* populations (Liance 1994; Collins & Paskewitz 1995; Phillips 2001; Reed et al. 2006). If it is also largely conserved across *P. chabaudi* clones, the additional use of this antigen would nicely contrast and complement the AMA-1 experiments in Chapters 2 and 3.

2) *Virulence Genes*. To date vaccine escape has always been thought to result from antigenic polymorphism (Crewther et al. 1996; Hodder et al. 2001; Mota et al. 2001; O'Donnell et al. 2001; Ekala et al. 2002; Healer et al. 2004; Polley et al. 2004; Cortes et al. 2005). However, in chapter 3 I speculate that virulence may play a role in vaccine efficacy and I describe ongoing experiments aimed at elucidating its role. The mechanisms underlying virulence are probably complex, involving a large number of genes associated with parasite phenotypes such as cell invasion, rosetting, cytoadherence and replication. Nonetheless, the discovery of genetic markers for parasite virulence or the discovery of the genetic determinants themselves would be highly desirable. If such discoveries were made, not only could virulence in the field be tracked, but experiments involving 'virulence manipulated' parasite lines would allow firm clarification of the role of virulence in vaccine efficacy (among other things) (Mackinnon & Read 1999b)

3) *Measuring Parasite Transmission*. It would also be exciting to explore the assertion that gametocyte density is positively associated with mosquito infection rate. Numerous other studies have found that this is not always the case and that neither gametocyte patency

(Boudin et al. 1993) nor density (Sattabongkot et al. 1991; Haji et al. 1996; Lensen et al. 1998) are correlated with infection rates. This discrepancy may be due to variation in the infectiousness of gametocytes throughout the life-span of an infection, such that a given density of gametocytes gives rise to very different infection rates in mosquitoes depending on the time point at which transmission is assessed (Dearsly et al. 1990; Butcher et al. 1996). Given that the gametocyte infectiousness may vary throughout the course of a malaria infection, the question arises as to how well gametocyte density on any particular day can predict the lifetime transmission success of a malaria parasite. To compliment these studies it would be of great interest to explore the infectiousness of vaccinated hosts versus naïve hosts. If immunity does indeed play a role in infectiousness, how does varying host immunity impact on transmission? Resolving these questions is fundamental for evolutionary biologists in order to evaluate whether it is possible to predict the selective advantage of particular parasite genotypes from point surveys.

4) *Mosquito Maternal Effects and Immunity.* Lastly, an exciting extension to the present work on parental effects in *Anopheles*, would be to study the role of maternal effects in determining resistance to malaria. The ability of hosts to resist and control infection is like all traits, dependant on the genotype, the environment and interaction between the two (Koella & Sorensen 2002; Lambrechts et al. 2005; Lambrechts et al. 2006b). Initial experiments could involve determining if maternal larval food level influences offspring infection rates. Trans-generational immune priming has now also been shown in a range of invertebrates including bumbles bees, daphnia and beetles (Carlier & Truyens 1995; Ravasi et al. 2002; Little et al. 2003; Sadd et al. 2005; Moret 2006). Understanding whether *Anopheles* mothers who experience an environment of immune stimulation, produce offspring with up regulated immune systems would greatly aid research into *Anopheles* resistance.

With these experiments complete, it may be possible to finally draw firm conclusions regarding the conditions that favour the evolution of virulence both generally and due to intervention strategies such as vaccination. However, generalities will need to be approached cautiously whilst data stems from only the malaria model system. In this regard, it is a concern that much of the experimental work on the evolution of virulence is focused on so few models systems. Ideally, this topic will, in the future be applied and tested in a many more host-parasite system, such that general principles of the evolution of harm are illuminated.

7. References

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8. Appendix – Published papers

Grech, K., Watt, K. & Read, A. F. 2006 Host-Parasite interactions for virulence and resistance in a malaria model system. *Journal of Evolutionary Biology* (19), 1620-1630.

Erratum: For the correct version of Table 1 see Table 2.1, Chapter 2.

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